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PRINCIPAL INVESTIGATOR: Robert E. Burke, M.D.

CONTRACTING ORGANIZATION: Columbia University
New York, NY 10032

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Robert E. Burke, M.D.

E-mail: rb43@columbia.edu

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14. ABSTRACT The molecular processes of programmed cell death (PCD) are important mediators of neural degeneration in Parkinson's disease (PD). The goal of this proposal is to examine in living animals the possible role of ER stress, a mediator of PCD, in dopamine neuron death. This is being done by the study of mice with targeted deletions of CHOP and caspase-12, mediators of ER stress-induced apoptosis. We have demonstrated that CHOP is universally expressed in neurotoxin model of parkinsonism. Assessment of the functional significance of CHOP expression by study of CHOP null mice has shown that in the 6OHDA model immature animals do not have diminished apoptosis, but mature ones do. The null mutation does not, however, protect dopamine neurons in the chronic MPTP model. We have assessed these models for other markers of ER stress. We have performed Northern and in situ hybridization anaylsis of BiP, and RT-PCR/Southern analysis of the XBP-1 splice variant. Neither is upregulated in these models. We therefore conclude that while CHOP is expressed and uniquely plays a functional role in the adult 6OHDA model, it may do so either in response to ER stress, or to oxidative stress. A complete manuscript reporting our findings has been submitted and conditionally accepted by the Journal of Neurochemistry. Since our last progress report, we have begun our studies of caspase-12 mice in the adult 6OHDA model.

15. SUBJECT TERMS
Apoptosis, programmed cell death, 6-hydroxydopamine, MPTP, Parkinson disease

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INTRODUCTION

There is a growing consensus that the molecular processes of programmed cell death (PCD) are important mediators of neural degeneration in Parkinson's disease (PD) and related disorders. However, while important recent advances in PD research have implicated both environmental and genetic factors in the pathogenesis of the disease, it has been unclear how these factors initiate the PCD cascade. The recent advances in our understanding of the genetic basis of PD, related to synuclein mutations which foster protein aggregation, and parkin mutations which result in a loss of functional ability to ubiquitinate difficult-to-fold proteins, have suggested a possible role for endoplasmic reticulum (ER) stress. In addition, it has been shown by analysis of gene expression in neurotoxin models in tissue culture, that ER stress may play a role in the PCD of dopamine neurons (3,4). The goals of this proposal are to examine in living animals whether CHOP, an upstream transcriptional mediator of ER stress-induced apoptosis, and caspase-12, a downstream mediator, play a role in PCD of dopamine neurons in neurotoxin models of parkinsonism. These goals will be achieved by studying mice with null mutations for these mediators. The sensitivity of the null animals to the induction of apoptosis in dopamine neurons will be examined in well-characterized and validated models of parkinsonism: intrastriatal injection of 6-hydroxydopamine in immature and adult mice, and chronic, systemic injection of MPTP in mice. Thus, the goals of our proposal remain the same as we had originally outlined.

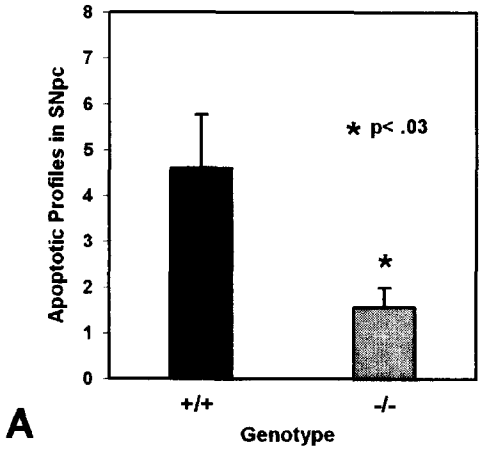
BODY

In our original proposal, we submitted preliminary data which indicated that ER stress is likely to occur and to be a mediator of programmed cell death in neurotoxin models of parkinsonism. Our colleague and collaborator on this proposal, Dr Lloyd Greene, had shown that the neurotoxin 6-hydroxydopamine (6OHDA) induces the expression of a number of mediators of an ER stress response in PC12 cells: ATF4, CHOP, BiP, phosphorylated PERK and others (3). A similar induction was noted on treatment of the cells with MPTP. He demonstrated that the ER stress response was likely to be mediating cell death in this culture model because sympathetic ganglion neurons derived from mice null for PERK, a mediator of a protective pathway in ER stress, were more sensitive to 6OHDA (3). Very similar findings were reported by Holtz and O'Malley for MN9D cells (4). The critical question which we therefore sought to address in this proposal is whether ER stress occurs in these neurotoxin models *in vivo*, and if so, whether it plays a role in mediating PCD. We submitted as preliminary data for this proposal immunohistochemical evidence that CHOP is expressed specifically in dopamine neurons of the SN in a model of intra-striatal injection of 6OHDA in immature rats. We showed that the time course of CHOP expression in these neurons in this model paralleled the induction of apoptosis. We also showed that CHOP is expressed in the SNpc in a chronic MPTP model, which induces apoptosis in dopamine neurons (5). We demonstrated that the expression of CHOP in dopamine neurons in association with apoptosis is specific for these neurotoxin models; CHOP expression is not observed in apoptosis associated with natural cell death, or axotomy-induced augmentation of natural cell death.

On the basis of these preliminary observations, we proposed three tasks to delineate the functional roles of CHOP and caspase-12, a downstream mediator of PCD in ER

stress, in dopaminergic neurotoxin-induced PCD in living animal models. We proposed to do this by studying the effects of null mutations for these mediators on dopaminergic cell death induced by 6OHDA and MPTP.

Task 1. **To determine if CHOP is a mediator of 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN) *in vivo*.**



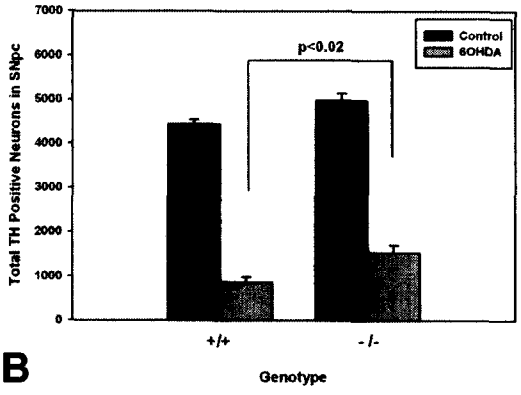


FIGURE 1. The CHOP null mutation protects from apoptotic cell death in the adult 6OHDA model. *A*, Wildtype (N=5) and CHOP homozygous null (N=6) adult mice were injected into the striatum with 6OHDA, and sacrificed six days later for TH immunostaining of the SN and counting of apoptotic profiles within the SNpc. The CHOP null animals demonstrated a 65% reduction in the level of apoptosis (p<.03, t-test).*B*, Wildtype (N=7) and CHOP null (N=8) adult mice were injected with 6OHDA and sacrificed 28 days later for TH immunostaining of serial sections for stereologic determination of the number of surviving dopaminergic neurons. In both genotypes, the 6OHDA injection led to a significant reduction in the number of SN dopamine neurons (p<.001, ANOVA; Tukey post-hoc). In the CHOP null animals, there was a 79% increase in the number of surviving neurons (p<.02, Tukey post-hoc). Nevertheless, the absolute magnitude of preservation of neurons (31%) was less than anticipated, based on a much greater level of suppression of death in the acute phase.

The work for this task has now been completed, and submitted as part of a full manuscript which has been accepted by the *Journal of Neurochemistry*. We have appended to this Annual Progress Report a copy of the submitted manuscript. All of the work completed for this task is presented in this full manuscript, but for the purposes of this Annual Report, we will touch upon the highlights:

In presenting this updated data, we would like to draw attention to a conclusion drawn in the "Memorandum for Military Operational Medicine Program", dated 09/08/04, under **TECHNICAL ISSUES**, wherein it was stated that we had "...disproved the CHOP component of the hypothesis upon which this proposal was based." This statement was based on our data through June 2004. Based on our full data set, reported herein, this statement is no longer correct. CHOP is a critical, essential mediator of dopamine neuron death in the adult 6OHDA model. We have shown that adult CHOP null mice have a diminished level of apoptosis in this model, and an augmented number of surviving neurons. At the time of our Annual Report in June 2004, we had shown that CHOP does not play a role in the 6OHDA model in *immature* mice. That is because, as we discuss in the manuscript, the induction of death in immature mice is predominantly due to an axotomy effect, in which CHOP does not play a role. In the

adult mice, death is entirely due to a toxic effect of 6OHDA, and in that context, CHOP is an essential mediator of neuron death. FIGURE 1A illustrates that adult CHOP null mice show a diminished level of induced apoptosis following intrastriatal injection of 6OHDA. We reported this in our last Progress Report. However, we now show in addition that this abrogation of apoptosis is lasting and significant because it results in an increased surviving number of dopamine neurons of the substantia nigra at 28 days postlesion (FIGURE 1B).

***Task* 2 To determine if CHOP expression is a general feature of neurotoxin-induced apoptosis in dopamine neurons of the substantia nigra *in vivo*.**

In relation to Task 2, we report in the manuscript that CHOP induction is a general feature specifically of neurotoxin-induced apoptosis in dopamine neurons, but it is not a general feature of all apoptosis in dopamine neurons. CHOP protein expression is observed in all of the 6OHDA and MPTP models tested, but it is not observed in apoptosis due to natural cell death, or that due to induction of natural cell death by axotomy (FIGURE 2).

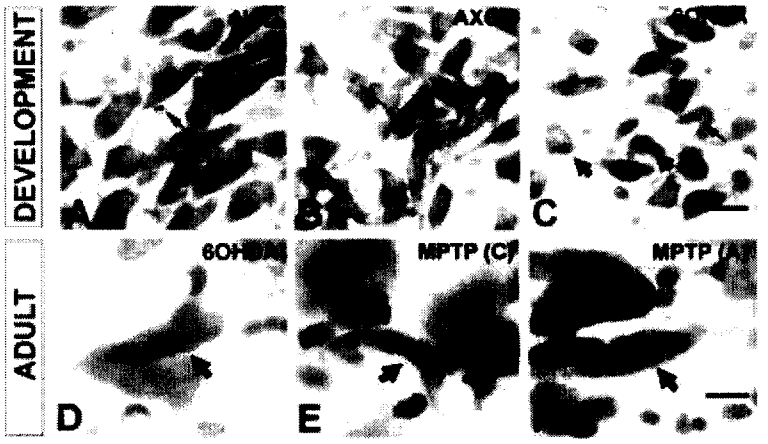


FIGURE 2. CHOP is expressed in neurotoxin models of induced death in SN dopamine neurons. *A,* CHOP expression does not occur in SN during the apoptotic postnatal natural cell death event. A representative field, showing a single apoptotic profile (arrow) in a PND14 rat is negative for CHOP immunostaining. *B,* The naturally occurring cell death event in SN can be augmented by postnatal axotomy of the dopaminergic axonal projection (1), as it is for many other developing neural projections (2). As for natural cell death, CHOP expression does not occur in this context, as shown by a representative field in a PND6 rat at 1 day postlesion. An apoptotic profile is shown (arrow). *C,* Unlike naturally occurring cell death and axotomy, cell death induced by 6OHDA results in the expression of CHOP in many neuronal profiles in the SNpc (broad arrowheads). In this model, CHOP-positive profiles rarely show basophilic apoptotic chromatin clumps (narrow arrow) (2% of instances). As discussed in the text, this rare association between CHOP expression and apoptotic nuclear morphology suggests that if CHOP is implicated in mediating death, it is likely to be an early participant, typically before morphologic change. Bar=20μm for A, B and C. *D,* A representative neuronal profile with a CHOP-positive nucleus (broad arrow) is shown at PLD6 following intrastriatal injection of 6OHDA in an adult mouse. *E, F,* CHOP nuclear staining is also observed in SNpc neurons following MPTP injection by either the chronic (C) or acute (A) regimens. Bar=10μm for D, E, F.

At the time of our June 2004 Annual Progress Report, we had shown that although CHOP expression is induced in the chronic MPTP model, it did not seem to play a critical role as a mediator of death, because CHOP null mice were not protected from induced death. In the past funding period, we have shown that CHOP null mice demonstrate equal levels of apoptosis as those observed in wildtype mice, and they show an equal degree of dopamine neuron loss. Thus, we have shown that there are fundamental differences in mechanisms of toxicity in the 6OHDA and MPTP models insofar as CHOP is concerned. To our knowledge, this is the first demonstration of a clear difference in mechanisms between these two models *in vivo*. This finding, however, is in keeping with the *in vitro* observations of Holtz and O'Malley, who noted a much more robust

induction of CHOP, and a broader ER stress response in the 6OHDA model in comparison to the MPTP model.

Task 3. **To determine if caspase-12 is a mediator of 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN)** ***in vivo.***

During the past year we have expanded our caspase-12 null colony, and we now have experiments underway to study the effect of the null mutation on sensitivity to death in the adult 6OHDA model.

Studies undertaken in response to Reviewer's comments

Our original proposal received a very fair and thorough review, and we decided that it is important to address one issue raised by the Reviewers. It was pointed out that expression of CHOP alone is not definitive evidence for the occurrence of an ER stress response. CHOP induction can occur under circumstances of oxidative stress and amino acid starvation, for example. The Reviewer therefore recommended that we examine other indicators of ER stress in our models. We have selected two. One is the BiP chaperone protein, which is often (but not always) upregulated in ER stress. The other is the ER stress splice variant of the transcription factor XBP-1, which is generally considered to be the most specific indicator of ER stress (personal communications, Drs David Ron; Kazutoshi Mori).

We examined the expression of BIP mRNA by Northern analysis in the chronic MPTP model at two time points: post-injection days 0 and 2. At neither time was BiP expression increased. In the adult 6OHDA model, we examined BiP expression by non-radioactive in situ hybridization. No induction was observed at 48 hours postlesion. Therefore there is no induction of this ER stress marker.

We also determined whether the XBP-1 422 bp splice variant could be identified in SN tissues in the acute or chronic MPTP or adult 6OHDA models. For this assessment, we used RNA derived from the kidney of a mouse treated with tunicamycin as a positive control. In the presence of this positive control, the XBP-1 splice variant was not detected in the chronic or acute MPTP models, or the 6OHDA model. There are two possible conclusions. It remains possible that ER stress is occurring in these models, but these markers thereof remain below the limit of detection in these studies conducted at the tissue (as opposed to cellular) level. The second possible conclusion is that CHOP is induced in these models not on the basis of ER stress, but rather some other cellular stress, such as oxidative stress.

KEY RESEARCH ACCOMPLISHMENTS

-We have demonstrated that the transcription factor CHOP, a mediator of ER stress-induced apoptosis, is expressed in the most important neurotoxin models of parkinsonism: 6OHDA-induced apoptosis in postnatal and mature rats and mice, and MPTP-induced cell death following acute or chronic administration.

-We have demonstrated that CHOP plays an essential functional role in the adult model of 6OHDA-induced apoptosis. However, we have also found that CHOP does not play a role as a mediator of neuron death in a postnatal model of 6OHDA-induced death, because in that model death is mediated primarily by an axotomy effect.

-We have demonstrated that although CHOP is expressed in both the acute and chronic MPTP models, it does not play a role as a critical mediator of neuron death, because mice null for CHOP are not protected. Thus, we have demonstrated that there is an important fundamental difference between the adult 6OHDA and MPTP models of Parkinson disease: CHOP mediates death in the former, but not in the latter. We do not know at this time which, if either, of these models is closely related to death processes in human PD. However, this demonstrated difference in these models will provide a useful basis on which to evaluate them, as we learn more about the biochemical correlates of cell death in the human disease.

-In spite of the expression of CHOP in these models, there is no further evidence at the tissue level for the expression of other markers of ER stress, including BiP and the 422 bp splice variant of the transcription factor XBP-1. It is therefore possible that CHOP expression is due to oxidative stress rather than ER stress in these models. Alternatively, it is possible that these studies, conducted at the tissue level, lacked the sensitivity to detect changes at the cellular level. Future investigations will depend on the creation of reagents which will make possible detection of these markers at the cellular level.

REPORTABLE OUTCOMES

The results summarized above have been reported as follows:

Silva RS, Oo TF, Jackson-Lewis VJ, Ryu E, Ron D, Przedborski S, Greene LA, Burke RE. The dopaminergic neurotoxins 6-hydroxydopamine (6-OHDA) and MPTP induce expression of CHOP (GADD153) in substantia nigra (SN) in vivo. Abstract, Society for Neuroscience, 2003.

Silva RM, Ries V, Oo TF, Yarygina O, Jackson-Lewis V, Ryu EJ, Lu PD, Stefan M. Marciniak, Ron D, Przedborski S, Kholodilov NG, Greene LA, Burke RE. CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an *in vivo* neurotoxin model of parkinsonism. J Neurochemistry, in press, 2005. (Attached).

The work done within this project on the role of CHOP in natural cell death in SN dopamine neurons was cited in the following recent review. Support by this award is acknowledged:

Burke RE. Ontogenic cell death in the nigrostriatal system. In: Unsicker K (Ed). The Dopaminergic Nigrostriatal System: Development, Physiology, Disease. Cell and Tissue Research. 2004, 318:63-72.

CONCLUSIONS

Based on our studies thus far, we firmly conclude that CHOP, a mediator of apoptosis due to ER stress, is upregulated in virtually all of the major neurotoxin models of parkinsonism. Our evidence indicates that CHOP is a functional mediator of apoptosis in the adult 6OHDA-induced model of parkinsonism. During this funding period, we have supported this conclusion by showing that CHOP null mice have a diminished level of apoptosis and an increased number of surviving dopamine neurons in the 6OHDA model.

Our results in the MPTP model indicate that CHOP is not a mediator of cell death in this model, in spite of the fact that it is robustly upregulated. During this funding period, we have supported this conclusion by showing that CHOP null mice have an equal level of apoptosis and an equal number of surviving dopamine neurons as compared to wildtype in the MPTP model.

We have found that two important markers of ER stress, the BiP chaperone protein, and the 422 bp splice variant of the transcription factor XBP-1, are not upregulated in the adult 6OHDA model, or the chronic or acute MPTP models. It is therefore possible that the upregulation of CHOP in these neurotoxin models is not mediated by ER stress, but rather another form of cellular stress, such as oxidative injury, which has been postulated to occur in both the 6OHDA and the MPTP models.

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CHOP/GADD153 Is a Mediator of Apoptotic Death in Substantia Nigra Dopamine Neurons in an *In Vivo* Neurotoxin Model of Parkinsonism

Robert M. Silva[1], Vincent Ries[1], Tinmarla Frances Oo[1], Olga Yarygina[1], Vernice Jackson-Lewis[1], Elizabeth J. Ryu[4], Phoebe D. Lu[5], Stefan M. Marciniak[5] David Ron[5], Serge Przedborski[1, 2, 3], Nikolai Kholodilov[1], Lloyd A. Greene[2, 3], and Robert E. Burke[1, 2,*]

Departments of Neurology[1], and Pathology[2],
The Center for Neurobiology and Behavior[3],
and The Institute of Human Nutrition[4]
The College of Physicians and Surgeons,
Columbia University, New York, NY 10032, U.S.A.

and

Departments of Medicine and Cell Biology[5],
The Skirball Institute of Biomolecular Medicine
New York University School of Medicine
New York, NY 10016

*Corresponding author:
Department of Neurology
Room 308, Black Building
Columbia University
650 West 168th Street
New York, NY 10032, U.S.A.
Tel: +1-212-305-7374
Fax: +1-212-305-5450
e-mail: rb43@columbia.edu

Abbreviations: 6OHDA: 6-hydroxydopamine; ABC: avidin-biotinylated-horseradish peroxidase complexes; ER: endoplasmic reticulum; MFB: medial forebrain bundle; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NRISH: non-radioactive in situ hybridization; PB: phosphate buffer; PBS: phosphate-buffered saline; PCD: programmed cell death; PCR: polymerase chain reaction; PD: Parkinson's disease; PLD: post lesion day; PND: postnatal day; SSC: saline sodium citrate; SN: substantia nigra; TBS: Tris buffered saline; TH: tyrosine hydroxylase;

ABSTRACT

There is increasing evidence that neuron death in neurodegenerative diseases, such as Parkinson's disease, is due to the activation of programmed cell death. However, the upstream mediators of cell death remain largely unknown. One approach to the identification of upstream mediators is to perform gene expression analysis in disease models. Such analyses, performed in tissue culture models induced by neurotoxins, have identified upregulation of CHOP/GADD153, a transcription factor implicated in apoptosis due to endoplasmic reticulum stress or oxidative injury. To evaluate the disease-related significance of these findings, we have examined the expression of CHOP/GADD153 in neurotoxin models of parkinsonism in living animals. Nuclear expression of CHOP protein is observed in developmental and adult models of dopamine neuron death induced by intrastriatal injection of 6-hydroxydopamine, and in models induced by MPTP. CHOP is a mediator of neuron death in the adult 6-hydroxydopamine model, because a null mutation results in a reduction in apoptosis. In the chronic MPTP model, however, while CHOP is robustly expressed, the null mutation does not protect from the loss of neurons. We conclude that the role of CHOP depends on the nature of the toxic stimulus. For 6-hydroxydopamine, an oxidative metabolite of dopamine, it is a mediator of apoptotic death.

Abbreviated title: CHOP/GADD153 Is a Mediator of Apoptotic Death

Key Words: apoptosis, programmed cell death, Parkinson's disease, substantia nigra, ER stress, oxidative stress

There is an emerging consensus that programmed cell death (PCD) is likely to play a role in neuron death in neurodegenerative disease (Yuan and Yankner 2000;Mattson 2000). For Parkinson's disease (PD), this consensus is based on studies in animal models and human postmortem material demonstrating either apoptotic morphology or immunohistochemical evidence for activation of caspases (reviewed in (Vila and Przedborski 2003)). One of the hallmarks of PCD is that in many contexts, it requires the transcription of genes that mediate cell death (Martin et al. 1988;Oppenheim et al. 1990). Therefore, a useful strategy to attempt to identify genes that mediate neuronal degeneration is to screen gene expression in models of disease. Such a strategy has been implemented for PD by performing serial analysis of gene expression in PC12 cells, a catecholaminergic cell line (Greene and Tischler 1976), treated with 6-hydroxydopamine (6OHDA), a neurotoxin which is an oxidative metabolite of endogenous dopamine (Kostrzewa and Jacobowitz 1974;Senoh and Witkop 1959). Among the upregulated transcripts identified by this analysis and of particular potential relevance to neuronal death was a striking induction of the transcription factor CHOP/GADD153 (Ryu et al. 2002). CHOP has been implicated as a mediator of apoptosis in the contexts of both endoplasmic reticulum (ER) stress (Matsumoto et al. 1996;Zinszner et al. 1998;Maytin et al. 2001;Kawahara et al. 2001;Gotoh et al. 2002;Oyadomari and Mori 2004) and oxidative stress (Mengesdorf et al. 2002;Guyton et al. 1996). In keeping with a possible role of either of these forms of cellular stress in mediating CHOP induction and neuron death, the analysis of gene expression also identified the

induction of many other genes involved in ER and oxidative stress (Ryu et al. 2002;Ryu et al. 2005).

A similar induction of CHOP was also observed by Holtz and O'Malley in a gene expression screen of neurotoxin models of parkinsonism (Holtz and O'Malley 2003). These investigators used Affymetrix gene arrays to screen dopaminergic MN9D cells following exposure to either 6OHDA or MPP^+, and noted that the most highly expressed transcript, for both neurotoxins, was that for CHOP (Holtz and O'Malley 2003).

These findings in gene expression screens performed *in vitro* are potentially relevant to human PD, because the classes of transcripts induced, those related to oxidative stress and ER stress, relate to important current hypotheses for pathogenesis. The possibility that the oxidative metabolism of dopamine may be injurious to dopaminergic neurons is one of the longest-standing hypotheses (Fahn and Cohen 1992). More recently, ER stress has been postulated to play a role. An important genetic cause of PD is loss of function mutations in *parkin* (Ishikawa and Tsuji 1996;Kitada et al. 1998). These mutations have been implicated in abnormal protein processing, because parkin is an E3 ubiquitin-ligase (Shimura et al. 2000), and as such it plays a role in targeting cellular proteins for destruction by the proteasome (Ciechanover 1998). One putative protein target of parkin, Pael-R, is a difficult-to-fold protein, and it has been postulated that its accumulation may result in dopaminergic neuron death due to ER stress (Imai et al. 2000;Imai et al. 2001).

The possible implications of these *in vitro* observations for the pathogenesis of PD depend on whether they generalize to the *in vivo* context. We have therefore investigated the expression of CHOP in several neurotoxin models of parkinsonism in living animals: substantia nigra (SN) dopamine neuron degeneration induced by intrastriatal injection of 6OHDA in both developing (Marti et al. 1997) and adult rodents (Sauer and Oertel 1994) and by both the acute (Heikkila et al. 1984) and chronic (Tatton and Kish 1997) systemic administration of MPTP. In addition we have sought to determine whether CHOP plays a functional role as an essential mediator of dopamine neuron death by examining the vulnerability of homozygous CHOP null mice.

Materials and Methods

Animals. For the study of postnatal rats, timed pregnant females were obtained from Charles River Laboratories (Wilmington, MA). The date of delivery was defined as postnatal day (PND) 1. For adult mouse studies utilizing the 6OHDA and MPTP models, C57BL/6 mice were obtained from Charles River. CHOP null mice were produced by homologous recombination to replace all of the CHOP coding sequence (except for the final 34 C-terminal residues) with the coding sequence for ß-galactosidase, containing a nuclear localization signal. The neomycin selection cassette was then removed by Cre recombinase. There was no detectable CHOP protein in cells and tissues derived from these animals (Fig. 1). These mice were backcrossed into the C57BL/6 strain five times before breeding for experiments. The CHOP null mice were genotyped by PCR

analysis of tail DNA using three-primer PCR analysis as described (Zinszner et al. 1998) with the modification that the primer to detect the mutant allele was based on the ß-galactosidase sequence, and produced a 300bp product.

Animal models. The models used in this investigation are summarized in the Table. The 6OHDA model in postnatal rats was performed as previously described (Marti et al. 1997). Briefly, rat pups at postnatal day PND7 were pretreated with 25mg/kg desmethylimipramine, anesthetized by hypothermia, and placed prone on an ice pack. 6-OHDA hydrobromide (Regis, Morton Grove, IL, USA) was prepared at 15 µg (total weight) / 1.0 µl in 0.9% NaCl / 0.02% ascorbic acid, and infused by pump (Harvard Apparatus, Holliston, MA, USA) at a rate of 0.25 µl / min for 4 minutes (total dose 15 µg). Postnatal mice were injected in a similar fashion except that the solution was prepared at a concentration of 20 µg/µL and infused for 2 minutes, for a total dose of 10 µg. For experiments in postnatal mice, littermate wildtype and heterozygote animals were examined in comparison to nulls. Adult mice were infused with a concentration of 5µg/µL at a rate of 0.5 µL/min for 8 minutes for a total dose of 20 µg. For experiments in adult mice, C57BL/6 adults were used as controls.

The medial forebrain bundle (MFB) axotomy model in postnatal rats was performed as previously described (El-Khodor and Burke 2002). Briefly, rat pups were anesthetized by hypothermia. Animals were positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) to conform with the neonatal brain atlas of Heller et al. (Heller et al. 1979). The MFB was transected by lowering a retractable wire knife (Kopf Instruments) through a skull burr hole 1.4 mm

posterior and 2.5 mm lateral to bregma to a ventral position of 6.5 mm below bregma.

For the acute MPTP lesion model, mice received four i.p. injections of MPTP-HCl (20 mg/kg free base; Sigma) dissolved in saline, 2 hours apart in one day as previously described (Teismann et al. 2003). Control mice received saline only. MPTP handling and safety measures were in accordance with our published guidelines (Przedborski et al. 2001). For the chronic MPTP model, mice received one i.p. injection of MPTP-HCl per day (30 mg/kg per day of free base) for 5 consecutive days as described (Tatton and Kish 1997).

All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University.

Immunohistochemistry. For CHOP immunoperoxidase histochemistry, animals were perfused intracardially first with 0.9% NaCl and then with 4% paraformaldehyde and 0.1 M phosphate buffer (PB). The brains were then removed and postfixed in the same fixative for 3 hours. Each brain was then cryoprotected in 20% sucrose for 24 hours. The brains were then rapidly frozen in isopentane on dry ice, and sections were cut in a cryostat at 30 µm. Sections were processed free-floating. After a phosphate-buffered saline (PBS) wash and treatment with PBS, 0.5% bovine serum albumen, and 0.1% Triton X-100, sections were incubated with rabbit anti-CHOP at 1:500 for 48 hr at 4°C. After a wash, sections were then incubated biotinylated protein A (prepared in this

laboratory) at 1:100 for 1 hr at room temperature. Sections were then incubated with avidin-biotinylated-horseradish peroxidase complexes (ABC; Vector Laboratories, Burlingame, CA) at 1:600 for 1 hr. After incubation with diaminobenzidine, sections were mounted onto subbed slides and counterstained with thionin. The primary antibody has been previously characterized and used for immunohistochemistry (Ron and Habener 1992;Zinszner et al. 1998). For immunofluorescence double-labeling for CHOP and tyrosine hydroxylase (TH) sections were collected into Tris buffered saline (TBS) and then treated with TBS/0.2% Triton/2% goat serum/2% horse serum. They were then incubated in the same solution with anti-CHOP (1:500) and mouse anti-TH (1:40) (Chemicon, Temecula, CA) for 48 hours at 4° C. The sections were then treated with Texas red horse anti-mouse (Vector Laboratories) at 1:75 and biotinylated goat anti-rabbit (Vector) at 1:75 for 1 hour at room temperature, followed by treatment with Fluor-avidin (Vector) at 1:100 for 1 hour. Sections were then mounted onto gelatin-coated glass slides and coverslipped with DAKO anti-fade medium. The sections were then examined by epifluorescence with a Nikon Eclipse 800 microscope.

For TH immunoperoxidase histochemistry animals were perfused as described above, and then postfixed in the same fixative for 1 week. Each brain was then cryoprotected in 20% sucrose for 24–48 hr and then rapidly frozen. A complete set of serial sections through the SN was then cut at 30 µm. Sections were saved individually in serial order at 4°C, and individual sections at regular intervals were then selected for TH immunostaining, in conformity with the fractionator method

of sampling (Coggeshall and Lekan 1996) (see below). Sections were processed free-floating, as described above for CHOP. The primary antibody was a rabbit anti-TH (Calbiochem, La Jolla, CA) at 1:1000. After treatment with biotinylated protein A and ABC, sections were mounted on subbed slides in serial order and thionin-counterstained.

Quantitative morphology. For the analysis of the time course of appearance of CHOP-positive nuclear profiles and apoptosis in the postnatal 6OHDA model in rats, counts were performed as we have previously described (Ganguly et al. 2004;Oo et al. 2003). CHOP-positive nuclear profiles were counted in identical fashion on the same sections.

The number of SN dopaminergic neurons in the lesion experiments with CHOP null and C57BL/6 control mice was determined by stereological analysis. A complete set of TH-immunostained serial sections, sampled as every fourth section through the SN, was analyzed by a stereological method for each animal. Each analysis was performed under blinded conditions on coded slides. For each animal, the SN on each side of the brain was analyzed. For each section, the entire SN was identified as the region of interest. Using StereoInvestigator software (Micro Bright Field, Inc., Williston, VT) a fractionator probe was established for each section. The number of TH-positive neurons in each counting frame was then determined by focusing down through the section, using a 100X objective under oil, as required by the optical dissector method (Coggeshall and Lekan 1996). Our criterion for counting an individual TH-positive

neuron was the presence of its nucleus either within the counting frame or touching the right or top frame lines (green) but not touching the left or bottom lines (red). The total number of TH-positive neurons for each SN on one side was then determined by the StereoInvestigator program. The total volume of the SN was also determined by the StereoInvestigator program for each brain on the basis of the sum of volumes derived from the area of each individual serial section and the tissue height represented by that section.

Northern analysis and non-radioactive in situ hybridization analysis (NRISH) of BiP. Rat BiP cDNA was subcloned into pCMS-EGFP (BD Biosciences, San Jose, CA) as described (Ryu et al. 2002) and used for creation of an antisense RNA probe. Northern analysis was performed as previously described (El-Khodor et al. 2001). Briefly, RNA was isolated from microdissected SN using Qiagen RNAeasy Mini kit. The RNA concentration of each sample was determined by measuring absorption at 260 nm on a GenQuant spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ). Twenty micrograms of each RNA was electrophoresed in 1.4% agarose-formaldehyde gel and transferred onto an Immobilon (+) membrane (Millipore, Bedford, MA). The hybridization was performed overnight at 68° C in Ultrahyb buffer from Ambion (Austin, TX). The membrane was then exposed to phosphorimager cassettes, scanned and analyzed by Image Quant software (Molecular Dynamics, Indianapolis, IN).

For NRISH, brains were rapidly removed from 6OHDA-injected adult mice at 48 hours post-injection, and rapidly frozen in embedding medium on dry ice. Sections (14 µm) were thaw-mounted on glass slides (Superfrost Plus, Fisher, Hampton, NH). For hybridization, sections were warmed on a slide warmer at 37°C for 20 minutes, and then fixed by immersion in 4% paraformaldehyde in 0.1 M PBS. After washing, sections were acetylated by treatment with acetic anhydride in triethanolamine. After another wash, sections were treated with a pre-hybridization solution as previously described (Burke et al. 1994) for 2 h at room temperature. Sections were then covered with hybridization solution and incubated overnight at 68° C in a humidified chamber. Hybridization solution contained the BiP riboprobe labeled with digoxigenin-UTP (1 µL/slide) (200-400ng/ml), prepared as per the manufacturer's instructions (Roche Diagnostics, Penzberg, Germany). The size and integrity of labeled probe were confirmed by gel electrophoresis. The same probe used for Northern analysis was used for the in situ hybridization. After a wash in 0.5 X saline sodium citrate (SSC) for 10 minutes, followed by a wash in 0.2X SSC at 68°C for 30 minutes, sections were incubated with an anti-digoxigenin antibody (Roche) at 1:5000 overnight at 4°C. After additional washes, sections were incubated with a solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega Corporation, Madison, WI) in a darkened humidified chamber overnight. Sections were then washed and coverslipped with DAKO aqueous mounting medium.

RT-PCR/Southern blot analysis of the XBP-1 splice variant. To perform Southern analysis of the XBP-1 splice variant, we first generated a DNA probe.

We performed reverse transcription using RNA isolated from mouse kidney after treatment with tunicamycin. We then performed PCR of the 422 bp region of mouse XBP-1 containing the site of the unconventional splice by using primers based on nucleotide number 363 (Accession Number BC029197) (5'-CCTTGTGG TTGAGAACCAGG-3') (forward) and nucleotide number 810 (5'-GAGGCTTGGTGTATACATGG-3') (reverse). The band containing the spliced DNA fragment of XBP-1 was isolated from an agarose gel, subcloned in the pGEM-T vector (Promega) and sequenced. The DNA fragment containing the site of the XBP-1 unconventional splice was isolated from this clone using SalI and NcoI restriction enzymes (Promega). This fragment was then used to generate a ^{32}P-labeled DNA probe with the Rediprime II Kit, random prime labeling system (Amersham Pharmacia Biotech, Piscataway, NJ). For XBP-1 splice variant Southern blot analysis, RNA was isolated from tissues using the QIAGEN RNAeasy Mini Kit, as described above. First strand cDNA was then synthesized from isolated RNA by RT system (Promega). PCR was performed individually with each cDNA sample using above primers with Taq polymerase from Roche. Ten micrograms of each DNA sample was electrophoresed in a 2% agarose gel. The DNA was then transferred onto a Hybond-N membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and hybridized with the XBP-1 DNA probe in Ultrahyb solution (Ambion, Austin, TX) overnight at 42°C, washed as recommended and then exposed to phosphorimager cassettes, scanned and analyzed by Image Quant software (Molecular Dynamics, Indianapolis, IN).

Statistical analysis. The time course of appearance of apoptotic and CHOP-positive profiles in the postnatal 6OHDA model was analyzed by ANOVA with a Tukey post-hoc analysis. Stereological determination of the number of SN dopaminergic neurons in the 6OHDA and MPTP lesion experiments was analyzed by ANOVA with a Tukey post-hoc analysis. The number of apoptotic profiles in wildtype and CHOP null adult mice in the 6OHDA model was analyzed by t-test. All statistical analyses were performed using SigmaStat software (SPSS Science, Chicago, IL).

Results

CHOP protein expression is induced in a developmental neurotoxin model of parkinsonism

We initially performed *in vivo* experiments in a rat developmental model in which the intrastriatal injection of 6OHDA results in the induction of death in dopamine neurons of the SN, exclusively with the morphology of apoptosis (Marti et al. 1997). In this model, the unilateral intrastriatal injection of 6OHDA resulted in the unilateral induction of CHOP protein expression, demonstrated by immunohistochemistry (Fig. 2A). On the side of injection, CHOP expression was observed only in the SNpc, the exclusive site of neuron death in this model (Marti et al. 1997). CHOP expression was characterized at a cellular level by performing double-label immunofluorescence for CHOP and TH, to identify dopaminergic neurons of the SN. This analysis revealed that CHOP was expressed predominantly in the nucleus (Fig. 2B,B'). To determine the cellular sites of CHOP expression within the SNpc, we examined 50 representative

CHOP-positive nuclear profiles among 6 sections derived from 2 animals. This analysis showed that all CHOP-positive nuclei were within TH-positive, dopaminergic neurons of the SNpc. Thus, there was a precise correlation at the cellular level between the neuronal population which undergoes death in this model, and CHOP expression (Fig. 2B). All of the CHOP- and TH-positive neuronal profiles identified by the double-labeling procedure had a normal neuronal morphology: abundant cytoplasm, with a polygonal shape, and tapered proximal dendrites. We know from previous studies of this model that the vast majority of dopamine neurons die (Marti et al. 1997). Therefore, CHOP-positive profiles (all of which were TH-positive) are exceedingly likely to be destined to die in this model. We therefore interpret the normal-appearing morphology to mean, that if CHOP is to be implicated as a death mediator, it is expressed early in the death process, before any morphologic change at the cellular level.

We investigated the time course of CHOP expression at the population level in this model. We recognize that since apoptosis occurs rapidly (Oppenheim 1991), and since at any given time of sacrifice of the animal there will be a heterogeneous population of dying cells in varying stages of the death process, this population analysis will not resolve the cellular sequence of events. Nevertheless, it is informative to determine whether, at the population level, the appearance of CHOP-positive profiles correlates with the appearance of apoptotic profiles. CHOP expression at the population level in this model correlated at most times with the induction of apoptotic death (Fig. 2C). The occurrence of the peak number of CHOP-positive nuclear profiles corresponded

precisely with the occurrence of the peak number of apoptotic profiles at postlesion day (PLD) 4. However, one exception to this correlation was that apoptosis was induced as early as PLD2, in the absence of any induction of CHOP, suggesting that an early component of apoptosis in this model is not associated with CHOP induction, as discussed further below.

Having demonstrated a co-localization between CHOP expression and the dopaminergic neuronal phenotype, and a temporal correlation between CHOP expression and apoptosis in the SN, we next examined the generality of the relationship in other developmental models, in which apoptosis occurs. During the postnatal development of SN dopamine neurons, there is naturally occurring cell death, exclusively with the morphology of apoptosis (Janec and Burke 1993;Oo and Burke 1997). Immunostaining for CHOP was performed on SN sections obtained from PND 14 rats (during the second phase of naturally occurring cell death). We examined 36 SN sections among N = 4 rats, and no instance of CHOP positivity was identified. Among these sections, 124 apoptotic profiles were identified, due to natural cell death (Fig. 3A). This naturally occurring cell death can be augmented by an axotomy lesion of the medial forebrain bundle during the postnatal period (El-Khodor and Burke 2002). Examination of 18 SN sections from three PND6 rats at 24 hours post-axotomy failed to reveal any CHOP-positive profiles (Fig. 3B). Among these sections numerous apoptotic profiles were identified in SN, as described (El-Khodor and Burke 2002), and sections from 6OHDA treated animals processed in parallel, were positive for CHOP (Fig. 3C). Thus, we conclude that in the postnatal

developmental period, CHOP protein expression is induced by the neurotoxin 6OHDA, but not by naturally occurring cell death or a physical lesion that augments it.

CHOP protein expression in adult neurotoxin models of parkinsonism

To investigate the expression of CHOP in adult neurotoxin models, we exclusively studied mice, to permit comparison between the 6OHDA model and the widely used MPTP mouse model of parkinsonism (Heikkila et al. 1984;Przedborski and Vila 2003). Adult mice injected into the striatum with 6OHDA demonstrated numerous CHOP-positive nuclei within neurons of the SNpc (Fig. 3D). For the study of MPTP effects on CHOP expression, we evaluated two dose regimens in common current use. Most widely used is an acute set of injections, 20mg/kg for four doses, two hours apart on a single day. This dosing regimen induces SN dopamine neuron death in the absence of apoptotic morphology (Jackson-Lewis et al. 1995). A second regimen utilizes a chronic set of injections, 30 mg/kg daily for 5 days (Tatton and Kish 1997), and results in neuron death with the morphologic characteristics of apoptosis. In both of these MPTP models, numerous CHOP-positive neuronal profiles were identified within the SN (Fig. 3E, F). In all of these adult contexts, positive nuclear CHOP expression was identified in neurons which otherwise appeared normal, suggesting, as previously discussed, that if CHOP is to be implicated as a death mediator in these models, then it is expressed prior to degenerative

morphologic changes. We conclude from these studies that CHOP is generally expressed in the SNpc in neurotoxin models of parkinsonism.

CHOP mediates neuron death in the adult 6OHDA model

Having demonstrated close relationships between CHOP expression and the death of SN dopamine neurons in these neurotoxin models we next sought to determine whether CHOP plays a critical functional role in mediating this death, as it has been shown to do in non-neuronal models of cell death due to ER stress (Zinszner et al. 1998) and oxidative stress. For this assessment, we compared the sensitivity of homozygous CHOP null mice to wildtype controls in their degree of sensitivity to neurotoxin-induced neuron death. In the postnatal 6OHDA model, we found that there was no difference between homozygous CHOP nulls and either heterozygous mice or wildtype controls in the degree of apoptosis among SN dopaminergic neurons induced by intrastriatal 6OHDA (Fig. 4). However, we recognized that in this model death is known to be mediated not only by the direct effect of the neurotoxin, but also, in the developmental period, by an "axotomy" effect due to destruction of dopaminergic terminals during a period of target dependence (Marti et al. 1997). Since we had shown directly that axotomy does not induce CHOP expression, we considered the possibility that this admixture of death mechanisms may obscure a role played by CHOP in death due to the neurotoxin. Such a possibility was also suggested by the time course analysis in Figure 2C, which showed an early apoptotic component in the absence of CHOP induction. We therefore examined the sensitivity of adult

CHOP null mice to intrastriatal injection of 6OHDA, as adult dopamine neurons do not have target dependence (Kelly and Burke 1996).

In adult mice, there was a clear protective effect of the homozygous CHOP null mutation (Fig. 5). CHOP null animals demonstrated a 65% reduction in the number of apoptotic profiles in the SNpc at the sixth postlesion day. To determine whether this reduction in the magnitude of neuron death resulted in a lasting protection from the neurotoxin, we examined the number of surviving TH-positive neurons in the SN at 28 days postlesion. This analysis revealed that the null mutation did provide a substantial, lasting protective effect; there was a 79% increase in the number of surviving TH-positive neurons in comparison to wildtype controls (Control: 857 ± 131; Null: 1531 ± 173 neurons per SN) (Fig. 5B). Nevertheless, the absolute magnitude of the protective effect in the nulls, expressed as 31% survival, while significantly greater than that in wildtype (19%, $p<.02$), was considerably less than anticipated based on a 65% suppression of apoptotic death in the acute period. In addition, at 28 days postlesion, there was no evidence for sparing of dopaminergic innervation of the striatum in the nulls. In the nulls, there was a 28.0±3.2 sparing of the optical density of TH-positive fibers within the striatum, as there was in wildtype controls (28.3±3.6).

CHOP mediates a cellular response to injury, but not neuron death, in the chronic MPTP model

Given that CHOP expression is induced in both the acute and chronic MPTP models, we sought to determine whether it plays a role as a death mediator, as it

does in the adult 6OHDA model. Since the role of CHOP as a death mediator has previously been identified in non-neuronal cells in the context of apoptosis (Zinszner et al. 1998), we examined its role in the chronic MPTP model, in which apoptosis has been identified (Tatton and Kish 1997). Based on our results in the adult 6OHDA model, demonstrating a disparity between the ability of the CHOP null mutation to protect from death in the acute period following the lesion as compared to the chronic period, we conducted separate assessments of both of these postlesion periods. We found that the CHOP null mutation provided a protective effect in the acute (PLD4) period following the chronic administration of MPTP. The CHOP null animals demonstrated only a non-significant trend for a decrease in the number of TH-positive SN neurons at this time whereas wildtype controls demonstrated a 65% decrease (Fig. 6A,B). However, this difference could not be attributed to a difference in the magnitude of apoptotic death between the two genotypes. While there was a trend towards fewer apoptotic profiles in these sections among the CHOP null mice (2.7±0.8 profiles/SN), this did not achieve significance in comparison to wildtype (5.2±1.1, $p>.1$, Tukey post-hoc). We therefore attribute the marked difference in number of TH-positive neurons between the two genotypes to the well-described suppression of TH phenotype following MPTP treatment (Jackson-Lewis et al. 1995). In keeping with this interpretation, in the chronic setting at 21 days postlesion, there was only a 36% decrease in TH neuron number following MPTP in the wildtype animals. This apparent increase in the number of TH-positive neurons between the acute and chronic lesion periods has previously been shown to be due to a recovery of phenotype (Jackson-Lewis et al. 1995). In the chronic period, in the

MPTP- treated mice, unlike the 6OHDA-treated mice, there was no protective effect of the null mutation on the number of surviving TH-positive neurons (Fig. 6C). This difference between the two models is in keeping with the lack of an effect of the null mutation on the magnitude of cell death in the acute period of the MPTP model, whereas there was a pronounced effect in the 6OHDA model. As would be expected from the lack of a protective effect of the null mutation on TH-positive neuron number, there was also no protective effect on striatal TH-positive fiber density (data not shown). We therefore conclude that in the chronic MPTP model, CHOP appears primarily to play a role in the loss of phenotype response which accompanies cellular injury, rather than in cell death, as it does in the 6OHDA model.

CHOP induction in neurotoxin models is not accompanied by changes in BiP mRNA expression, or the appearance of the XBP-1 splice variant

The induction of CHOP alone cannot be taken as compelling evidence for the occurrence of the ER stress response, because CHOP can be induced by other cell stressors, such as oxidative stress, arsenite exposure and amino acid limitation (Mengesdorf et al. 2002;Jousse et al. 1999;Entingh et al. 2001;Bruhat et al. 1997). Therefore, to determine whether the induction of CHOP observed in these models was indicative of the broader ER stress response, we examined the mRNA expression of an ER-resident chaperone, BiP (also know as Grp78) (Kaufman 1999;Gething 1999). Induction of BiP mRNA has previously been shown to occur *in vitro* in conjunction with CHOP induction upon exposure of neuronal cells to 6OHDA (Ryu et al. 2002;Holtz and O'Malley 2003). In addition,

we assessed nigral tissue by PCR for the presence of a splice variant of the transcription factor x-box binding protein-1 (XBP-1) (Yoshida et al. 2001;Calfon et al. 2002) a specific marker for the unfolded protein response.

Northern analysis of SN tissue from mice treated according to the chronic MPTP regimen, on the last day of injection (PLD0) or two days after the final injection, failed to demonstrate any change in BiP mRNA in comparison to saline-treated controls (not shown). To conduct an analysis of BiP mRNA expression at the SNpc regional and cellular levels in the adult 6OHDA model, we performed NRISH. As previously reported by others for normal rat (Little et al. 1996) we observed widespread constitutive expression of BiP mRNA in brain (not shown). However, we did not observe any induction in SNpc, at the regional or cellular level, following unilateral intrastriatal 6OHDA injection at PLD2. A similar analysis of MPTP-treated mice failed to show any difference in BiP mRNA expression in SNpc in comparison to saline-treated controls (not shown).

Southern analysis of PCR reaction products for the XBP-1 unspliced and spliced variants was performed with the inclusion of a positive control derived from renal tissue of tunicamycin-treated mice, in which the ER stress response has previously been demonstrated (Zinszner et al. 1998). This analysis was performed for SN tissues derived from 6OHDA-treated mice, at 1 and 3 days postlesion, and for tissues derived from mice treated with MPTP according to both the acute and chronic regimens. In no instance was the XBP-1 splice variant identified in SN tissues, in spite of its clear presence in tunicamycin-

treatment renal tissue. We conclude that in spite of the induction of CHOP protein in these models, there is no additional biochemical evidence of an unfolded protein response, using these methods at the tissue level.

Discussion

The hypothesis that PCD plays a role in neural degeneration in PD rests principally on two forms of evidence. First, in rodent neurotoxin models, there is histologic and biochemical evidence for activation of PCD mediators, such as the caspases, and functional evidence from genetic and pharmacologic studies (reviewed in (Vila and Przedborski 2003)). Second, while traditional morphologic assessments of human PD postmortem brains for apoptosis have been controversial, there has been growing evidence for activation of caspases (Hartmann et al. 2000;Hartmann et al. 2001;Viswanath et al. 2001). While this evidence validates PCD as a target for the development of neuroprotective therapeutics, much remains unknown, particularly about upstream mediators, which would make attractive therapeutic targets (Yuan and Yankner 2000).

The identification of CHOP as a markedly upregulated transcript following the treatment of catecholaminergic cell lines with dopaminergic neurotoxins (Ryu et al. 2002;Holtz and O'Malley 2003) and with rotenone, a mitochondrial Complex 1 inhibitor (Ryu et al. 2002) is of particular interest, because, as a transcription factor, it would be likely to play an upstream regulatory role. In keeping with that possibility, a gene activated by CHOP, DOC6, is homologous to gelsolin, a mediator of cytoskeletal collapse during apoptosis (Wang et al. 1998). CHOP is

also of particular interest in relation to PD because it has been implicated as an apoptotic mediator in the setting of oxidative stress (Mengesdorf et al. 2002;Guyton et al. 1996), which has been long postulated to play a role in PD (reviewed in (Fahn and Cohen 1992)), and ER stress (Matsumoto et al. 1996;Zinszner et al. 1998;Maytin et al. 2001;Kawahara et al. 2001;Gotoh et al. 2002;Oyadomari and Mori 2004), which has likewise recently been implicated in this disease (Imai et al. 2000;Imai et al. 2001).

We have determined that CHOP is expressed in neurotoxin animal models of parkinsonism. In a developmental model of apoptosis induced in dopamine neurons of the SN by the intrastriatal injection of 6OHDA (Marti et al. 1997), there was robust induction of CHOP protein expression exclusively within the SNpc. At a cellular level CHOP expression was nuclear, as expected for a transcription factor, and exclusively within dopaminergic neurons. CHOP expression was also observed in neurotoxin models in the adult setting, following intrastriatal 6OHDA, and either acute or chronic systemic MPTP exposure. In these adult models, as in the developmental 6OHDA model, CHOP expression was strictly within the SNpc at a regional level, and within the nucleus of otherwise normal-appearing neurons at a cellular level. CHOP expression, however, is not a universal feature of apoptosis in dopamine neurons; in the developmental setting, it is observed neither during naturally occurring cell death (Janec and Burke 1993;Oo and Burke 1997), nor with augmentation of this death by axotomy (El-Khodor and Burke 2002). On the basis of classic neurotrophic theory (Clarke 1985) the naturally occurring cell death event, and its augmentation by axotomy, would be

considered to be regulated by the availability of neurotrophic support. Our observations that CHOP is not induced in these conditions, but it is by neurotoxic insults, are comparable to those of Ryu et al *in vitro* (Ryu et al. 2002), who noted that CHOP is induced by neurotoxins, but not by neurotrophic withdrawal.

The principal finding of these investigations was that adult CHOP null mice were resistant to apoptotic death in SN dopamine neurons induced by the intrastriatal injection of 6OHDA. We considered the possibilities that this reduction may be due to a change in the time course of apoptosis, or the rate of clearance of apoptotic profiles in the null mice, rather than an actual reduction in the eventual magnitude of the death event. We therefore assessed the final surviving number of SN DA neurons at PLD28, and found that they were increased, indicating that the null mutation did in fact reduce the magnitude of death. We therefore conclude that CHOP is an important functional mediator of apoptosis in the 6OHDA model. Given that CHOP is highly expressed prior to any morphologic change in dopamine neurons destined to die in this model, we postulate that CHOP is likely to be an early mediator in the death process. Although the CHOP null mutation was protective in this model, the degree of preservation of SN dopamine neurons in absolute terms, 31%, was less than anticipated based on a 65% suppression of apoptotic death in the early postlesion period. This discrepancy suggests that some of the death which ultimately occurs in the CHOP nulls is delayed. There are two possible explanations for this delay. First, it is possible that death mediators other than CHOP eventually come into play (Ryu et al. 2005) and bring about the loss of the majority of dopamine neurons.

Second, it is possible that in these non-temporally regulated nulls, compensatory changes have taken place to provide alternate death pathways. These two possibilities are not mutually exclusive.

In view of the ability of the CHOP null mutation to provide neuroprotection in the adult 6OHDA model, the question arises why it did not also provide protection in the postnatal model, in which CHOP expression is clearly induced. Our interpretation of this difference rests on our previous demonstrations that during the first two postnatal weeks, SN dopamine neurons are dependent on interactions with their target, the striatum, as envisioned by classic neurotrophic theory (Clarke 1985), whereas in adults they are not (Macaya et al. 1994;Kelly and Burke 1996;Stefanis and Burke 1996). Therefore, during this postnatal period, the death of SN dopamine neurons following their destruction of their nerve terminals with 6OHDA is likely to be mediated by an "axotomy" effect as well as a direct neurotoxic effect. This interpretation is supported not only by the aforementioned studies of the developmental time course of striatal target dependence, but also by our demonstrations that the postnatal 6OHDA model is characterized by two cellular patterns of caspase activation: a perinuclear pattern, as observed in naturally occurring cell death (Jeon et al. 1999;Oo et al. 2002;El-Khodor and Burke 2002), and a cytoplasmic pattern, observed in direct neurotoxic injury (Jeon et al. 1999;Oo et al. 2002). Given this likelihood of an axotomy effect in the postnatal 6OHDA model, and based on our demonstration herein that CHOP is not expressed following developmental axotomy, we would anticipate that a functional role for CHOP would be difficult to discern in the postnatal 6OHDA lesion.

MPP+, the toxic metabolite of MPTP, induced CHOP expression in *in vitro* models (Ryu et al. 2002;Holtz and O'Malley 2003). MPTP treatment *in vivo* likewise induced the expression of CHOP protein, but in the chronic MPTP model, unlike the 6OHDA model, the CHOP null mutation did not significantly diminish the level of apoptosis or increase the number of surviving neurons. The null mutation did, however, prevent the loss of TH immunoreactivity in the period early after the MPTP injections. We interpret this relative preservation of TH immunoreactivity, in the absence of protection from cell death, to be attributable to protection from the loss of phenotype which is well-documented in this (Jackson-Lewis et al. 1995) and other neuronal injury models (Wooten et al. 1978). We conclude that while CHOP plays a role in regulating cellular phenotype in the MPTP model, it is not likely to play a role as an important death mediator. This difference in the role of CHOP, between the 6OHDA and MPTP models in living animals is consistent with the observations made *in vitro* by Holtz and O'Malley (Holtz and O'Malley 2003). Following treatment with 6OHDA, they observed a greater induction of CHOP, and a more general induction of other ER stress markers than with MPTP treatment.

To determine whether the CHOP induction observed in these neurotoxin models was specifically due to ER stress, we assayed mRNA expression of the ER-resident chaperone BiP (Kaufman 1999;Gething 1999) and the splice variant of XBP-1 (Yoshida et al. 2001;Calfon et al. 2002). In none of the models was there a change observed in BiP mRNA expression, or the appearance of the XBP-1

splice variant. These results were not unexpected for the MPTP model in view of the *in vitro* results which showed no induction of BiP or XBP-1 by MPP+ (Holtz and OMalley 2003). However, these results were unexpected for the 6OHDA model, as both prior *in vitro* studies had demonstrated clear evidence for a full ER stress response induced by 6OHDA (Ryu et al. 2002;Holtz and O'Malley 2003). There are two principal interpretations of these negative results. First, it is possible that CHOP induction in the 6OHDA model in living animals is not part of a full ER stress response, the *in vitro* results notwithstanding. It is well established that 6OHDA produces oxidative stress (Heikkila and Cohen 1973;Cohen and Heikkila 1974). It is therefore possible that its induction of CHOP in living animals is mediated principally by cellular oxidative stress (Mengesdorf et al. 2002;Guyton et al. 1996). Alternatively, it is possible that the studies of BiP and the XBP-1 splice variant, which were performed at the tissue level, lacked the sensitivity to detect changes, which, for CHOP, were detected at the cellular level by immunohistochemistry. Thus, our inability to detect other markers for ER stress in the 6OHDA model does not permit us to definitively conclude that it is not present.

We conclude that these investigations performed in living animals are largely supportive of the *in vitro* results suggesting the possibility of a role for CHOP in the neurodegeneration associated with PD. We find, as predicted from these gene expression screens, that CHOP is expressed in diverse neurotoxin models of dopamine neuron death. These observations support the validity of the *in vitro* screens for genes of potential relevance to disease. In addition, we find that

CHOP can play a role as a mediator of cell death, depending on the context; in the 6OHDA model CHOP is a necessary death mediator. The context specificity of CHOP is an important feature, because it suggests that it may be possible in designing neuroprotection strategies to target disease-related death pathways without interfering with other apoptotic pathways that may be important for survival of the organism.

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TABLE. Models used to assess the role of CHOP/GADD153 in apoptosis in SN dopamine neurons.

TREATMENT	SPECIES	AGE	ROUTE	MORPHOLOGY OF CELL DEATH
None (Natural cell death)	Rat	Developmental	N.A.	Apoptosis
Axotomy	Rat	Developmental	N.A.	Apoptosis
6OHDA	Rat or Mouse	Developmental	Intrastriatal	Apoptosis
6OHDA	Mouse	Adult	Intrastriatal	Apoptotic and non-apoptotic
MPTP	Mouse	Adult	I.P., Acute	Non-apoptotic
MPTP	Mouse	Adult	I.P., Chronic	Apoptotic and non-apoptotic

Abbreviations: N.A.: not applicable; I.P.: intraperitoneal

FIGURE LEGENDS

Figure 1. Absence of CHOP protein expression in CHOP null mice.

Immunoblot of nuclear extract of untreated and tunicamycin-treated (2mcg/ml, 6 hours) wildtype and CHOP-/- cells blotted with antisera reactive with CHOP, ATF4 (a positive control) and p75 a ubiquitously expressed nuclear protein that serves as a loading marker. No protein CHOP expression is observed in CHOP null cells after tunicamycin treatment. The antibody to ATF4 was raised against a full-length bacterially expressed fusion protein and is characterized in Ron and Habener 1992. The p75 band was detected by an antiserum to Drosophila protein, described in Immanuel et al. 1995.

Figure 2. Localization and time course of CHOP expression following developmental 6OHDA lesion in postnatal rats.

A, Low power photomicrographs at PLD6 of the substantia nigra contralateral (Control: Con) and ipsilateral (Experimental: Exp) to an intrastriatal injection of 6OHDA in a PND7 rat. CHOP protein expression is demonstrated by immunoperoxidase staining without a counterstain. CHOP-positive nuclei therefore appear as punctate brown profiles at this power. On the contralateral Control side (A'), there is an absence of staining. On the ipsilateral Experimental side, numerous CHOP-positive profiles are observed within the SNpc (B'). No positive profiles were observed in the SNpr or in the midbrain dorsal to the SNpc.

B, Double immunofluorescence labeling for CHOP and TH in the SNpc at PLD4 following intrastriatal injection of 6OHDA in a PND7 rat. TH immunostaining is demonstrated by Texas Red (A'), CHOP by fluorescein (B'), and the merged image is shown in C'. CHOP immunostaining was predominantly nuclear. Following 6OHDA injection, CHOP staining was observed strictly within TH-positive, dopaminergic profiles of the SNpc. Note that CHOP positive profiles appear normal morphologically; there is no apparent change in neuronal shape or proximal dendrites in comparison to adjacent, CHOP-negative, TH-positive neurons. Bar in C'=10μm.

C, Time course for the appearance of apoptotic and CHOP-positive profiles in SN following intrastriatal injection of 6OHDA in PND7 rats. A total of 24 rats

were studied: N=4 at PLD0 and 2; N=5 at PLD4 and 6; N=6 at PLD8. CHOP-positive and apoptotic profiles were counted in the same sections from each animal, as described in Methods. The number of CHOP-positive profiles reached a peak at PLD4 (**p <.02 vs PLD0, 2 and 8; ANOVA, Tukey post-hoc). The number of apoptotic profiles also reached a peak at PLD4 (*p<.05 vs PLD0 and 8; ANOVA, Tukey post-hoc). However, the time of induction for the two types of profile differed at PLD2; for apoptotic profiles the number at PLD2 was induced and not significantly different from the number at peak, whereas for CHOP profiles, there was no induction at PLD2. As discussed in the text, this difference may suggest that there are non-CHOP-dependent, as well as CHOP-dependent, mechanisms of cell death in this model.

Figure 3. CHOP is expressed in neurotoxin models of induced death in SN dopamine neurons. CHOP immunoperoxidase histochemistry was performed on free-floating sections, as described in Methods, with rabbit anti-CHOP (Zinszner et al. 1998) at 1:500 for 48 hours, followed by thionin counterstain.

A, CHOP expression does not occur in SN during the apoptotic postnatal natural cell death event. A representative field, showing a single apoptotic profile (arrow) in a PND14 rat is negative for CHOP immunostaining.

B, The naturally occurring cell death event in SN can be augmented by postnatal axotomy of the dopaminergic axonal projection (El-Khodor and Burke 2002), as it is for many other developing neural projections (Oppenheim 1991). As for natural cell death, CHOP expression does not occur in this context, as shown by a representative field in a PND6 rat at 1 day postlesion. An apoptotic profile is shown (arrow).

C, Unlike naturally occurring cell death and axotomy, cell death induced by 6OHDA in PND7 rat results in the expression of CHOP in many neuronal profiles in the SNpc (broad arrowheads). In this model, CHOP-positive profiles rarely show basophilic apoptotic chromatin clumps (narrow arrow) (2% of instances). As discussed in the text, this rare association between CHOP expression and apoptotic nuclear morphology suggests that if CHOP is implicated in mediating death, it is likely to be an early participant, typically before morphologic change. Bar=20μm for A, B and C.

D, A representative neuronal profile with a CHOP-positive nucleus (broad arrow) is shown at PLD6 following intrastriatal injection of 6OHDA in an adult mouse.

E, F, CHOP nuclear staining is also observed in SNpc neurons following MPTP injection in adult mice by either the chronic (C) or acute (A) regimens. Bar=10μm for D, E, F.

Figure 4. The CHOP null mutation does not protect from induction of apoptosis in the developmental 6OHDA model. A total of 20 PND6 mice (wildtype N=5; heterozygous N=10; null N=5) received a unilateral intrastriatal 6OHDA injection and were sacrificed at PLD4 for the determination of apoptotic profiles within the TH-immunostained substantia nigra, as described in Methods. In all three genotypes, there was a robust induction of apoptosis, as previously described for rats (Marti et al. 1997) (ANOVA $p<.001$ for the 6OHDA effect). There were, however, no differences among the genotypes for this effect.

Figure 5. The CHOP null mutation protects from apoptotic cell death in the adult 6OHDA model.

A, Wildtype (N=5) and CHOP homozygous null (N=6) adult mice were injected into the striatum with 6OHDA, and sacrificed six days later for TH immunostaining of the SN and counting of apoptotic profiles within the SNpc. The CHOP null animals demonstrated a 65% reduction in the level of apoptosis ($p<.03$, t-test).

B, Wildtype (N=7) and CHOP null (N=8) adult mice were injected with 6OHDA and sacrificed 28 days later for TH immunostaining of serial sections for stereologic determination of the number of surviving dopaminergic neurons. In both genotypes, the 6OHDA injection led to a significant reduction in the number of SN dopamine neurons ($p<.001$, ANOVA; Tukey post-hoc). In the CHOP null animals, there was a 79% increase in the number of surviving neurons ($p<.02$, Tukey post-hoc). Nevertheless, the absolute magnitude of preservation of neurons (31%) was less than anticipated, based on a much greater level of suppression of death in the acute phase.

Figure 6. The CHOP null mutation provides early protection from loss of phenotype, but not from neuron death, in the chronic MPTP model.

A, Wildtype and CHOP null adult mice were injected with saline or MPTP (30mg/kg/d) for five days (N=4 each group, except wildtype saline N=3) and sacrificed at 4 days after the last dose for immunostaining and stereologic determination of TH-positive neuron number. Remarkably, there was minimal apparent effect in the CHOP nulls treated with MPTP. The wildtype mice showed a 65% decrease in number of TH-positive profiles. This difference could not be attributed to a change in the magnitude of apoptosis, as discussed in the text.

B, Representative low power photomicrographs demonstrating the resistance of SN dopamine neurons in CHOP null mice to the early effect (4 days post-lesion) of MPTP in the chronic injection model. These sections are derived from mice studied by stereologic analysis of TH-positive neuron number, shown in Panel A. Bar=300μm.

C, Wildtype and CHOP null mice were injected with saline or MPTP (N=4-5 each group) and sacrificed at 21 days following the final injection for TH immunostaining and stereology. At this late postlesion day, when the acute suppression of phenotype has recovered, it is apparent that there has been only a 36% loss of SN dopamine neurons in wildtype mice. While there was a trend for a reduction in the number of neurons lost in the CHOP null mice (29% loss) this did not achieve significance ($p>.5$, Tukey post-hoc).

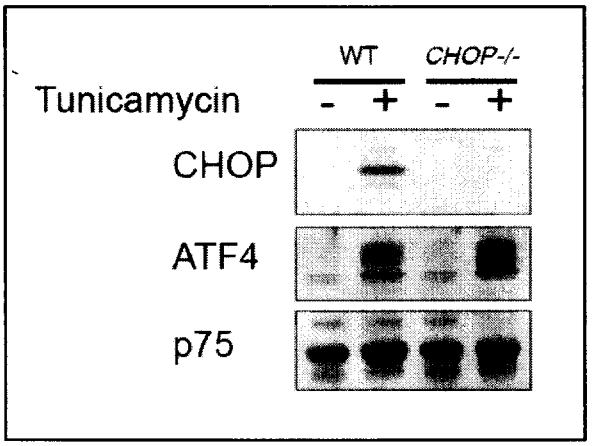

FIGURE 1

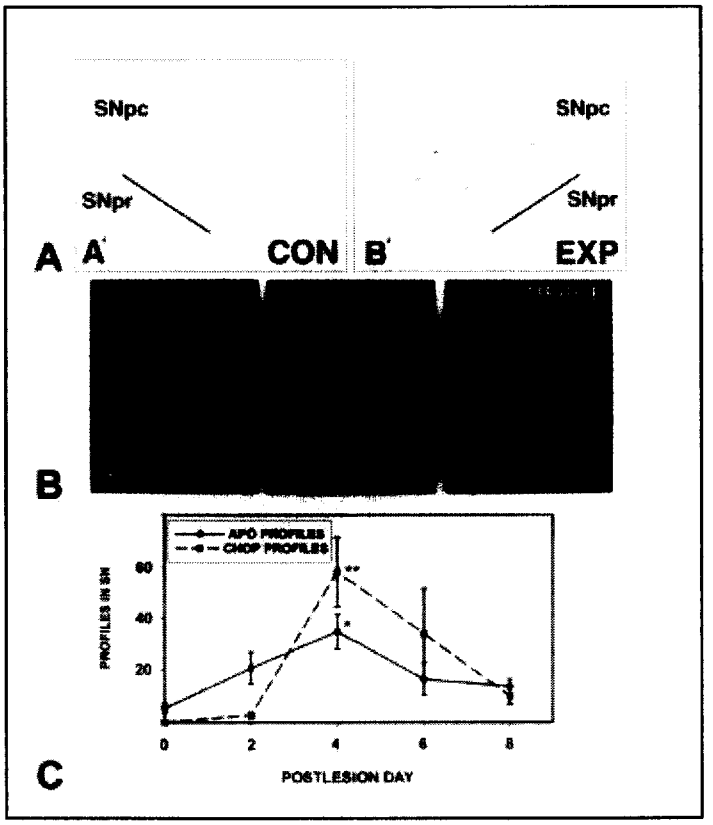
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FIGURE 2

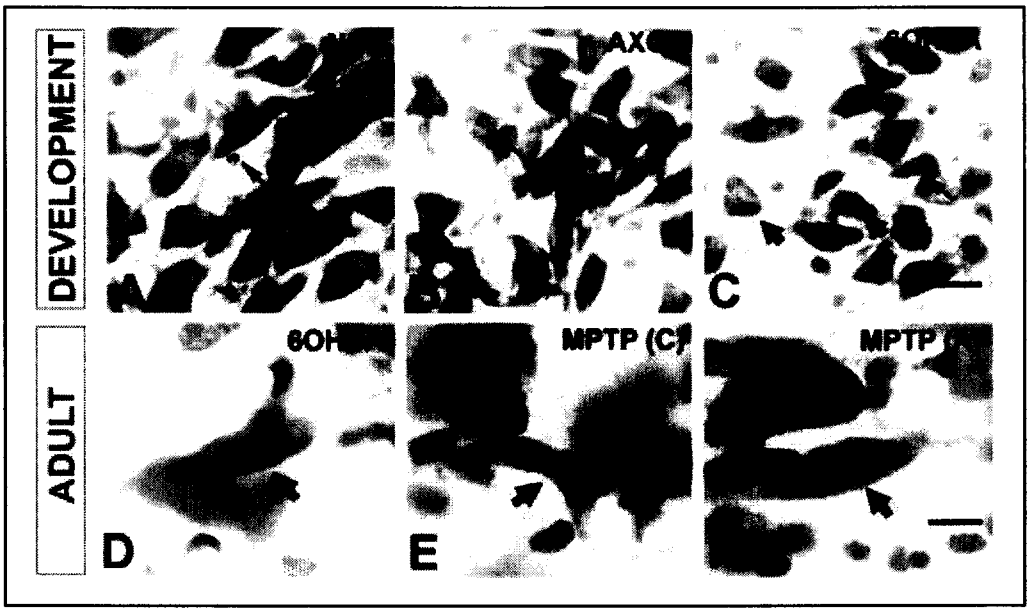


FIGURE 3

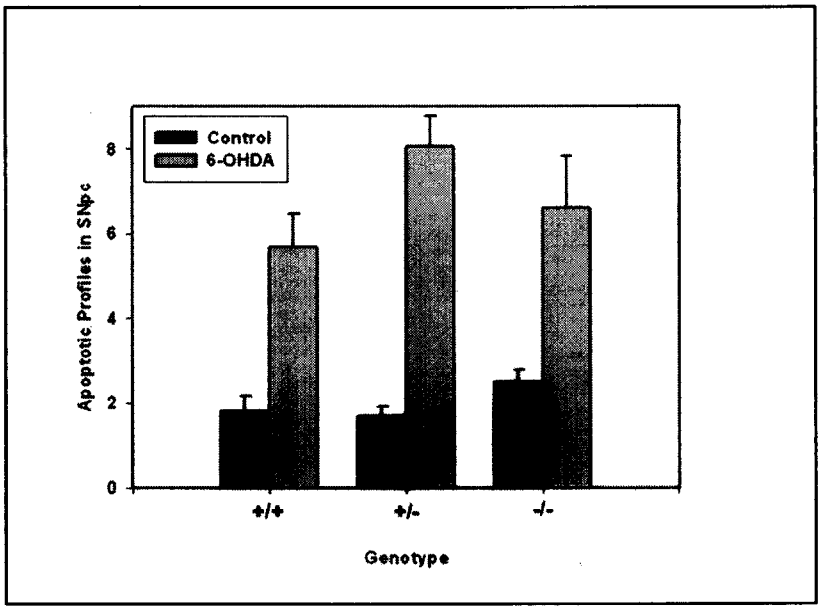
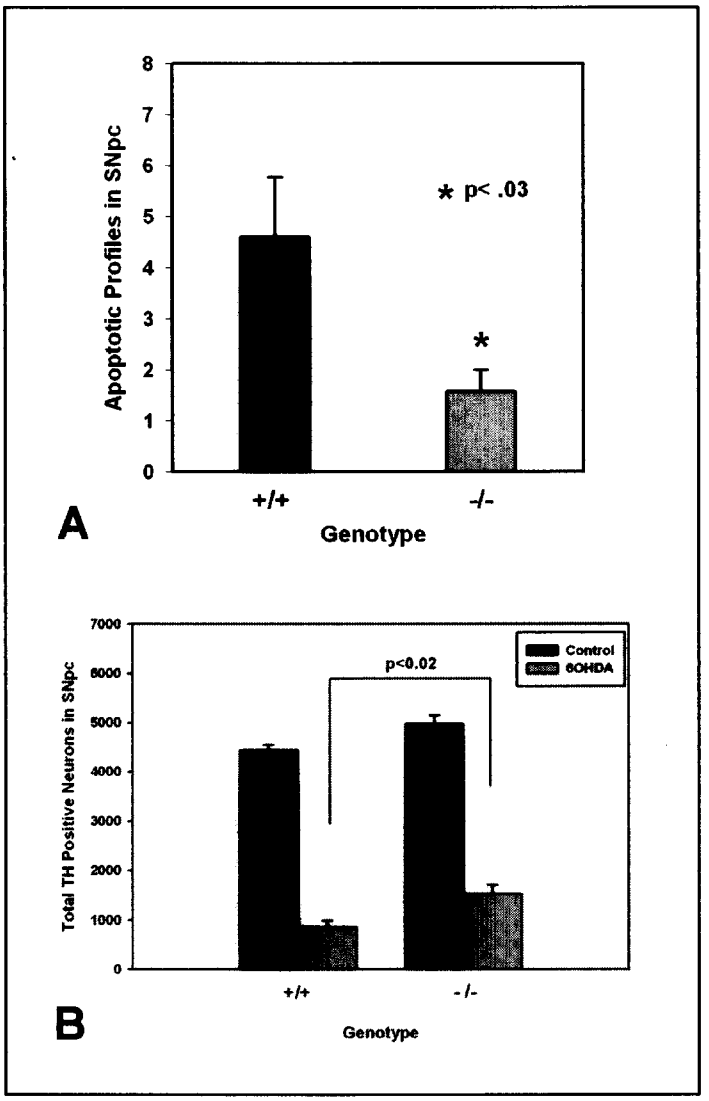


FIGURE 4


Apoptotic Profiles in SNpc
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A
Total TH Positive Neurons in SNpc
7000
6000
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p<0.02
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6OHDA
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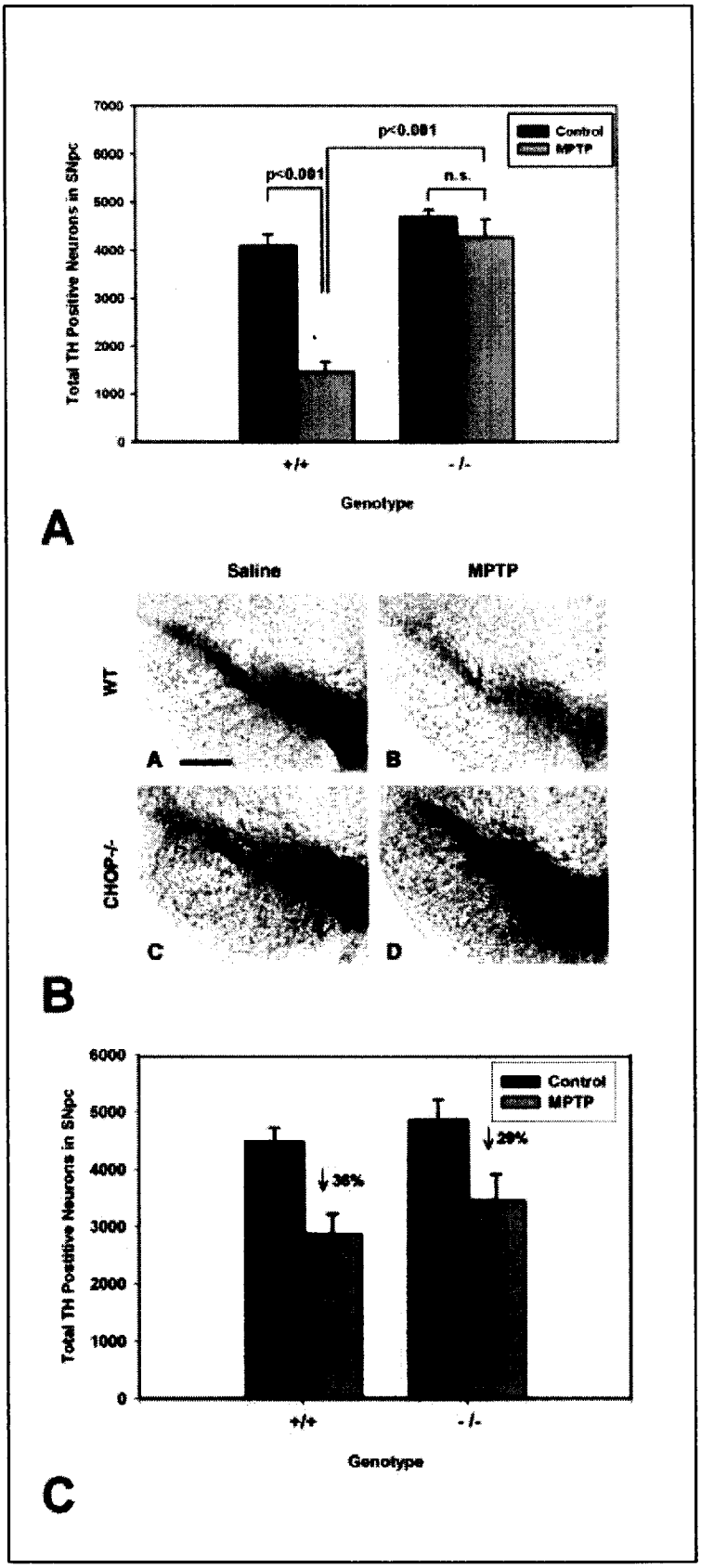
FIGURE 5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Total TH Positive Neurons in SNpc
7000
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+/+
-/-
Genotype
B
Saline
MPTP
WT
CHOP-/-
A
B
C
D
C
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6000
5000
4000
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29%
+/+
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Genotype

FIGURE 6

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REVIEW

Robert E. Burke

Ontogenic cell death in the nigrostriatal system

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Abstract Like most neural systems, dopamine neurons of the substantia nigra undergo apoptotic natural cell death during development. In rodents, this occurs largely postnatally and is biphasic with an initial major peak just after birth and a second minor peak on postnatal day 14. As envisioned by classic neurotrophic theory, this event is regulated by interactions with the target of these neurons, the striatum, because a developmental target lesion results in an augmented natural cell death event with fewer nigral dopamine neurons surviving into adulthood. Until recently, the striatal target-derived neurotrophic factors providing developmental support of dopamine neurons were unknown, but there is now growing evidence that glial-cell-line-derived neurotrophic factor (GDNF) serves as a physiologic limiting neurotrophic factor for these neurons during the first phase of natural cell death. During this phase, intrastriatal injection of GDNF diminishes the natural cell death event and neutralizing antibodies augment it. Sustained overexpression of GDNF in the striatum throughout development in a unique double transgenic mouse model results in an increased number of dopamine neurons surviving the first phase of natural cell death. However, this increase does not persist into adulthood. Therefore, other factors or mechanisms must play important roles in the determination of the mature number of nigral dopamine neurons. Further elucidation of these mechanisms will be important in the development of neuroprotective and cell replacement therapies for Parkinson's disease.

Keywords Dopamine neurons · Cell death · Nigrostriatal system · Glial-cell-line-derived neurotrophic factor · Parkinson's disease

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R. E. Burke (✉)
Department of Neurology, The College of Physicians and Surgeons, Columbia University,
650 West 168th Street,
New York, NY 10032, USA
e-mail: rb43@columbia.edu
Tel.: +1-212-3057374
Fax: +1-212-3055450

R. E. Burke
Department of Pathology, The College of Physicians and Surgeons, Columbia University,
650 West 168th Street,
New York, NY 10032, USA

Introduction

An important principle of neural development is that neuronal populations are created in excess numbers during embryogenesis and undergo a natural cell death event that determines their final adult number (Purves and Lichtman 1985; Clarke 1985; Oppenheim 1991). There is much evidence that the magnitude of this natural cell death event is regulated by competition among members of the neuronal population for support by their target. The precise nature of the competition for support may be diverse (Purves and Lichtman 1985) and in forms yet to be defined at a cellular level, but extensive evidence indicates that one component of this competition is limiting protein neurotrophic factors provided by the target (Oppenheim 1991; Clarke 1985; Barde 1989). Any neuron within a developing population that fails (1) to contact its target with an axon, (2) to take up the relevant trophic factor, and (3) successfully to transport its survival signal retrogradely will undergo natural cell death. Classically, it has been proposed that this competitive strategy, resulting in the regulation of the death event, serves two principle purposes: first, correctly to match numbers of neurons in a projecting neuronal population and its target, and second, to eliminate any projecting neurons with incorrect target connections (Clarke 1985).

It is important to recognize that these classic concepts of neurotrophic theory rest largely on experiments performed on neural systems with peripheral targets, such as sympathetic ganglion neurons, ciliary ganglion neurons, motor neurons, and the isthmo-optic nucleus (Purves and Lichtman 1985; Clarke 1985), and far less is known about

central neurons with targets within the central nervous system. Evidence exists that central neurons are also likely to match their numbers to the size of their targets; this is best exemplified by the matching of cerebellar granule cell numbers with the numbers of their target Purkinje cells in chimeric mice with mutations affecting the latter (Wetts and Herrup 1983; Herrup and Sunter 1987). However, much remains to be discovered about the mechanisms and neurotrophic factors involved in regulating developmental natural cell death events of central neurons. The mesencephalic dopaminergic system is of particular interest because of its importance to human neurologic and psychiatric disease, including Parkinson's disease, addictive behaviors, and schizophrenia. Certainly, the propensity to develop these disorders is likely to be influenced by the mature number of mesencephalic dopamine neurons and the developmental events that regulate that number.

Natural cell death in dopamine neurons of the substantia nigra

A natural cell death event occurs in the substantia nigra (SN) in both rats (Janec and Burke 1993) and mice (Jackson-Lewis et al. 2000). The morphology of this death event has been identified as apoptotic by light microscopy, electron microscopy, TUNEL labeling, and by immunostaining for the activated form of caspase-3 (Janec and Burke 1993; Jackson-Lewis et al. 2000). Useful light-microscopic techniques, for ease of identification and quantification of these apoptotic profiles, include a thionin stain (Clarke and Oppenheim 1995) and suppressed silver stain (Gallyas et al. 1980), both of which clearly label distinctive intranuclear chromatin clumps, characteristic of apoptosis (Fig. 1). Whereas in other developmental settings, other non-apoptotic morphologies of cell death have been identified, including cytoplasmic and autophagic forms (Clarke 1990), these forms have not been identified in SN by either electron microscopy or the suppressed silver stain. The latter is a sensitive technique for screening large number of sections at the light-microscope level for alternate morphologies of cell death (Oo et al. 1996), and no morphology other than apoptosis has been identified in the developing SN.

In order to determine the time course of natural cell death for dopamine neurons of the SN, we have used immunohistochemistry for tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, to define the dopaminergic phenotype, combined with a thionin counterstain to identify apoptosis (Fig. 2A). Profiles with apoptotic chromatin clumps and TH-positive cytoplasm are dopaminergic at a cellular level. However, as neurons undergo apoptosis, they are stripped of their cytoplasm and decrease their expression of phenotypic markers, such as TH (Freeman et al. 1994). Therefore, many dopaminergic neurons undergoing apoptosis would be expected no longer be identifiable as such, based on stains for cytoplasmic phenotype markers. To retain information about such profiles, which are the majority within the SN pars compacta (SNpc), we quantify apoptotic profiles (identified by thionin) that satisfy a regional criterion; i.e., they are in close proximity to TH-positive neurons within the SNpc (Fig. 2B). Counts of SNpc apoptotic profiles with a TH-positive cytoplasm correlate closely in number and time of appearance with counts of profiles meeting this regional criterion (Fig. 3; Oo and Burke 1997).

The cell death event in the SN begins on embryonic day 20 (E20) in rats and reaches a peak on PND2, defined as the day after birth. The event reaches a nadir by PND8-PND12 but then resurges on PND14, before ceasing on PND28 (Fig. 3). Thus, the event is largely postnatal and is biphasic, with the major phase being the first, just after birth. The time course of this event is similar in mouse

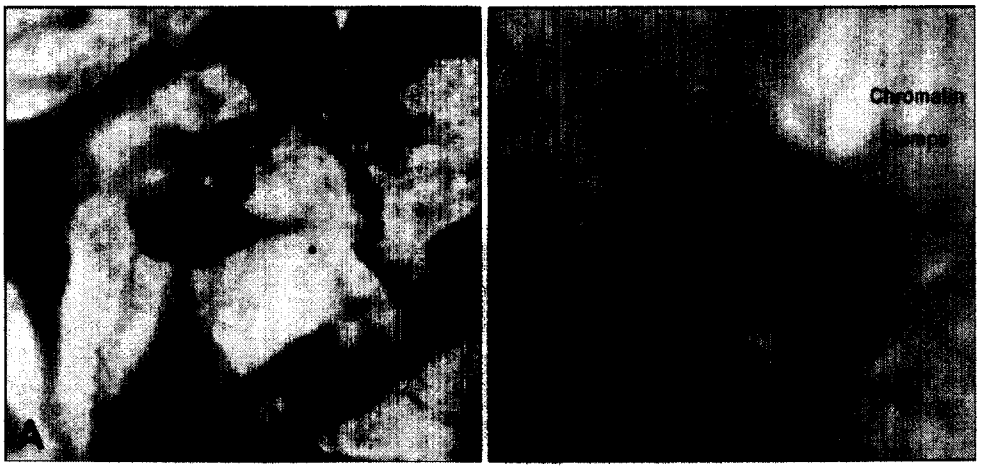

Fig. 1A, B Apoptotic profiles in SN pars compacta (SNpc) during postnatal natural cell death. **A** Thionin stain at postnatal day 8 (PND8). Within the nucleus of this neuron are three intensely, homogeneously stained, round, chromatin clumps with sharp, clearly defined edges. These chromatin clumps are highly characteristic of apoptosis at the light-microscope level. Note that this profile, in spite of the presence of apoptotic chromatin in its nucleus, has some preservation of neuronal morphology, including a polygonal shape and a dendrite. **B** Suppressed silver stain of an apoptotic profile at PND2. An example of an intensely argyrophilic chromatin clump is indicated by an *arrow*. These images are adapted from Janec and Burke (1993)

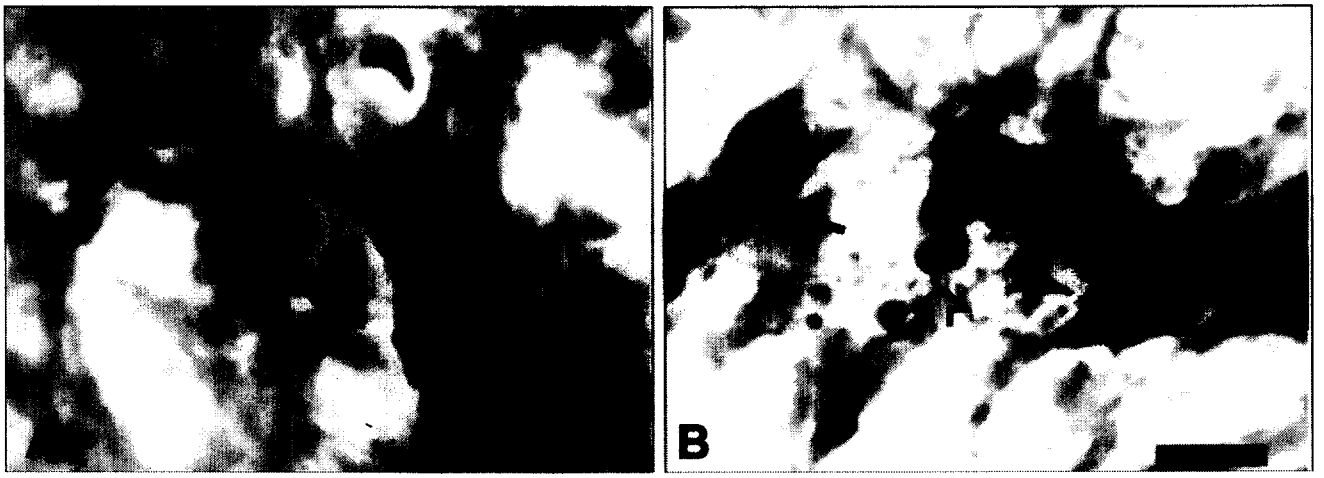

Fig. 2A, B Apoptotic profiles in the SNpc during postnatal natural cell death identified by TH immunostaining and thionin counterstaining. **A** TH immunostaining is demonstrated by brown chromogen reaction product. A dopaminergic neuron is identified by TH-positive cytoplasm (*arrowheads*). Within this neuron, apoptotic chromatin clumps are identified in the nucleus (*curved arrow*). This profile therefore meets cellular criteria for being a dopaminergic apoptotic profile. **B** An apoptotic profile (*open wide arrow*) is identified within 15 μm of two adjacent TH-positive neurons (*arrows*). This profile therefore meets regional criteria for being in the SNpc. Adapted from Oo and Burke (1997)

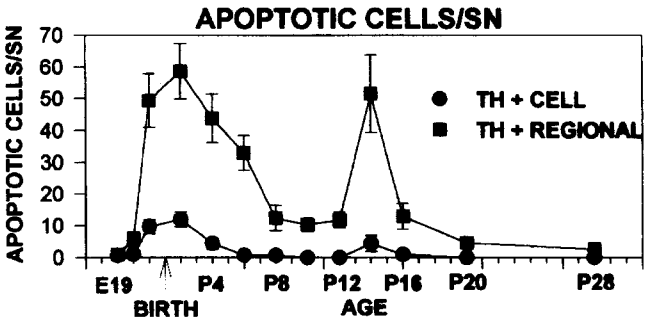

Fig. 3 The time course of natural cell death in dopaminergic neurons of the SNpc (*E* embryonic, *P* postnatal). Natural cell death among dopamine neurons is largely postnatal and biphasic, with an initial major peak just after birth and a second minor peak at PND14 (Oo and Burke 1997)

(Jackson-Lewis et al. 2000). It should not be assumed, based on these data, that apoptosis occurs in the developing mesencephalic dopaminergic population exclusively within the perinatal and postnatal periods. Our analysis began at E19, so it remains possible that there is an earlier independent natural cell death event affecting newborn dopamine neurons. This perinatal and postnatal event does, however, occur after mitosis has ceased among dopamine neurons (Marchand and Poirer 1983; Lauder and Bloom 1975) and so has an irreversible effect on their final adult number.

The mechanistic basis for this biphasic time course is unknown, but distinct developmental events are probably involved. The first phase of natural cell death occurs at a time when the nigral dopaminergic innervation of the striatum becomes complete; it is partial and localized to the ventrolateral striatum at E18 and essentially complete by PND4 (Kalsbeek et al. 1992). The precise nature of the competitive event during the first phase of natural cell death is also unknown but certainly relates, at least in part, to a limiting striatal supply of glial-cell-line-derived neurotrophic factor (GDNF), as will be discussed below. Little is known about the mechanisms controlling the second phase of cell death. Several important developmental events occur within the nigro-striatal system at this time: there is a maximal level of production of synapses within the striatum (Hattori and McGeer 1973) and within the SNpc (Lauder and Bloom 1975), the latter indicating the maturation of afferent projections to the SNpc. As discussed below, SN dopamine neurons appear to retain their dependence on their target at this time, but GDNF does not appear to play a regulatory role as a limiting factor.

The question often arises regarding the magnitude of the natural cell death event in dopamine neurons: how many are lost? This is not known. It is not possible to use information about the number of apoptotic profiles in sections during this event to derive the number of neurons that are lost, because the duration of a given apoptotic profile in living brain is not known. If one tries to determine the number of neurons lost by simply counting Nissl-stained neuronal profiles, there are methodologic difficulties, because not all dopamine neurons of the SN are in a single well-delineated somatotopic location, unlike motor neuron nuclei of the brainstem and spinal cord. Even where they are most concentrated, in the SNpc, they are not the only population present; some gamma-aminobutyric-acid-ergic neurons are also found here (Jackson-Lewis et al. 2000). If one tries to count the number of TH-positive neurons, there is also a methodological concern, because the level of phenotypic markers within each cell increases during this developmental period (Coyle 1977), making the number of profiles detected by immunohistochemistry steadily increase, even as these neurons undergo natural cell death. When such counts of TH-positive profiles have been performed (Jackson-Lewis et al. 2000;

Tepper et al. 1994), they show decrements in TH-positive neuron number, confirming a natural cell death event, but these changes almost certainly underestimate the number of neurons lost.

Limited information exists about the molecular pathways mediating programmed cell death during natural cell death in dopamine neurons. At the risk of oversimplification, programmed cell death mechanisms can be thought of as mediated by three major interacting pathways: the intrinsic pathway, through which caspase-9 is activated by cytochrome c release from mitochondria; the extrinsic pathway, in which the interaction of ligands with cell surface receptors leads to the activation of caspase-8; and the endoplasmic reticulum (ER) stress pathway, which is postulated to result in activation of caspase-12 (Mehmet 2000). During natural cell death, ER stress seems unlikely to be involved, as there is no expression of CHOP, an important mediator of apoptosis during ER stress (Zinszner et al. 1998; unpublished observation). To date, there is no information available about the possible role of the extrinsic pathway. Several lines of evidence indicate that components of the intrinsic pathway play a role. Members of the Bcl-2 family take an important part in controlling the release of cytochrome c and other cell death mediators from mitochondria, with the ensuing activation of the caspase cascade leading to cell death (Kluck et al. 1997; Scorrano and Korsmeyer 2003). When Bcl-2 is overexpressed specifically within catecholamine neurons under the control of the TH promoter in transgenic mice, there is suppression of natural cell death in SNpc (Jackson-Lewis et al. 2000). This suppression results in a 30% increase in the adult number of SN dopamine neurons. Homozygous Bax null mice show diminished levels of apoptotic natural cell death; however, the null mutation does not result in an increased number of SN dopaminergic neurons in adult animals (Vila et al. 2001). This result suggests that other pro-apoptotic members of the Bcl-2 family may be able to mediate death in the absence of Bax. In normal rats, an increase occurs in the ratio of Bax to Bcl-2 in the nigra during the natural cell death period, supporting the possibility of a role for these proteins in regulating the natural cell death event (Groc et al. 2001). Caspases of the intrinsic pathway are involved in natural cell death of dopamine neurons. During development, the activated form of caspase-9 can be identified within apoptotic profiles in the SNpc (Ganguly et al. 2004). The activated form of the downstream effector, caspase-3, can also be identified in apoptotic profiles (Jeon et al. 1999), as can protein cleavage products of caspase-3 (Oo et al. 2002).

Fas/Fas ligand (FasL) interactions, which initiate programmed cell death through the extrinsic pathway, have been implicated in natural cell death and induced programmed cell death in motorneurons (Raoul et al. 1999), and evidence has been obtained that these interactions utilize neuronal nitric oxide synthase (NOS; Raoul et al. 2002). As mentioned, the possible role of the extrinsic pathway, and Fas-FasL interactions in particular, remains largely unexplored for natural cell death in dopamine neurons. However, a direct investigation of a possible role for NOS has revealed no effect of the inhibitors L-NAME or 7-NI on levels of natural cell death in these neurons (Groc et al. 2002).

Natural cell death in dopamine neurons of the SN is regulated by interactions with striatal target

Many early studies performed in vitro suggested that the developing dopaminergic neurons of the SN were supported, both in their viability and differentiation, by striatal preparations. Prochiantz et al. (1979) first demonstrated that primary dissociated striatal cells grown in co-culture with embryonic mesencephalic dopamine neurons enhanced their differentiation. Hemmendinger et al. (1981) showed that embryonic mesencephalic dopamine neurons formed the appropriate number and type of axons in co-aggregate culture only in the presence of the appropriate target tissue. Subsequently, this group also reported that striatum in co-culture with mesencephalic dopamine neurons increased their viability (Hoffmann et al. 1983). Tomozawa and Appel (1986) demonstrated that a soluble factor purified from rat striatum was able to support the viability and differentiation of embryonic mesencephalic dopamine neurons.

Studies in vivo have also provided evidence that the striatal target influences the development of SN dopamine neurons. We have observed that an axon-sparing lesion of the striatum, made during development, results in a smaller number of SNpc dopamine neurons in adulthood (Burke et al. 1992). This decrease occurs in the absence of any acute injury to striatal dopaminergic terminals (Coyle and Schwarcz 1976) or any direct injury to the nigra itself. We have subsequently shown that, as classic neurotrophic theory would predict, this loss of striatal target during development is associated with a striking augmentation of the nigral apoptotic cell death event (Macaya et al. 1994; Fig. 4). Since striatal neurons may provide not only retrograde support to dopamine neurons, but also afferent projections to them, we have selectively assessed the role of retrograde support by ablating dopaminergic terminals by intra-striatal injection of the catecholaminergic neurotoxin 6-hydroxydopamine (6OHDA). This lesion would be expected to abrogate striatal retrograde support of the nigro-striatal projection but spare striatal afferents to nigra. Like a striatal target lesion, this lesion results in a striking augmentation of the nigral apoptotic death event (Marti et al. 1997; Fig. 4) and so, retrograde influences are likely to regulate the death event, at least in part. We have also shown that, as classic neurotrophic theory would again predict, an axotomy of the nigro-striatal axons within the medial forebrain bundle results in an induction of apoptosis (El-Khodor and Burke 2002) (Fig. 4).

Both the excitotoxic striatal target lesion model and the 6OHDA model show a developmental dependence in their ability to augment apoptotic death. They both produce a maximal effect during the first two postnatal weeks (Marti et al. 1997; Kelly and Burke 1996). Thus, the develop-

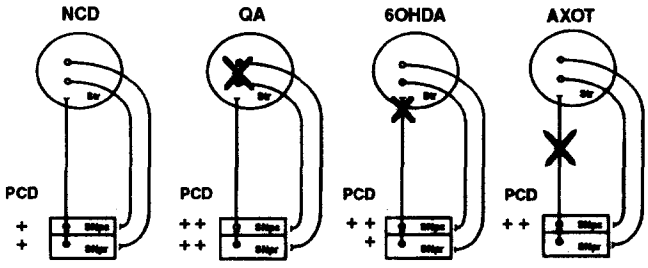

Fig. 4 Natural cell death (*NCD*) in dopamine neurons is regulated by target interactions in vivo. NCD occurs in both SNpc and SN pars reticulata (SNpr; unpublished observations) with an exclusively apoptotic morphology of programmed cell death (*PCD*). The anatomic connections between the *SNpc*, *SNpr*, and striatum (*Str*) are highly simplified in this diagram: dopaminergic neurons, with their cell bodies in *SNpc*, project to the striatum. Neurons of both the *SNpc* and *SNpr* receive afferent projections from the striatum. As predicted by classic neurotrophic theory, the apoptotic *PCD* event in *SNpc* is augmented by an axon-sparing striatal excitotoxic lesion made with quinolinic acid (*QA*; Macaya et al. 1994). Disruption of dopaminergic target contact by selective lesion with 6-hydroxydopamine (*6OHDA*) also augments *PCD* (Marti et al. 1997), although in this model, there is also likely to be a toxic effect of the 6OHDA. As predicted by classic neurotrophic theory, axotomy (*AXOT*) induces apoptotic cell death (El-Khodor and Burke 2002)

mental period of maximal death induction by these two lesions, which interfere with target support of developing dopaminergic neurons, is synchronous with the period of their natural cell death event. Such a synchrony between the period of target dependence and natural cell death has been observed for other systems (Clarke 1985).

In all of these models, the light-microscopic morphology of cell death induced by the lesion is apoptotic and no different from that observed during natural cell death. In this respect, dopamine neuron developmental death differs from some other systems in which the morphology of induced death may differ from the natural form (Purves and Lichtman 1985). It is important to mention however that, although apoptotic profiles visualized by thionin and silver staining do not differ among these models and natural cell death, differences in morphology can be observed between the 6OHDA model and the others when profiles are visualized by immunostaining for the activated form of caspase-3 and its protein cleavage products (Jeon et al. 1999; Oo et al. 2002). In the 6OHDA model alone, immunostaining is observed in the cytoplasm of some apoptotic neurons and in the nucleus; in natural cell death, striatal target lesion and axotomy, it is strictly nuclear. In the 6OHDA model, therefore induced apoptosis is probably observed not only because of loss of target support, but also because of a direct toxic effect.

GDNF is a candidate striatal target-derived neurotrophic factor for SN dopamine neurons

Since its discovery, GDNF has been considered to be a candidate neurotrophic factor for SN dopamine neurons (Lin et al. 1993). In support of the possibility that it may serve as a striatal target-derived factor, its mRNA is expressed in striatum, most abundantly during the early postnatal period (Schaar et al. 1993; Stromberg et al. 1993; Blum and Weickert 1995; Choi-Lundberg and Bohn 1995; Golden et al. 1999; Cho et al. 2003). We have recently shown that developmental striatal expression of GDNF mRNA at a cellular level is exclusively within medium-sized striatal neurons; there is no expression within glia (T. F. Oo et al., submitted). GDNF is also expressed within the postnatal striatum at the protein level (Lopez-Martin et al. 1999). We have shown that cellular protein expression can be identified in rare medium-sized striatal neurons, but not in glia. Most GDNF immunostaining has been identified within the striatal neuropil, some of which is positive for TH. In this cellular location, we propose that GDNF is likely to be undergoing retrograde transport, based on several observations: (1) specific retrograde transport of GDNF by the dopaminergic nigrostriatal system has been demonstrated (Tomac et al. 1995); (2) GDNF mRNA is more abundant in striatum than SN (T.F. Oo et al., submitted); and (3) GFRα1 mRNA is more abundant in SN than striatum (J. Cho et al., submitted). The abundant expression of GFRα1 mRNA in SNpc and the signaling tyrosine kinase Ret offer further support for GDNF as a possible neurotrophic factor for developing SN dopamine neurons (Widenfalk et al. 1997; Yu et al. 1998). The principal evidence marshaled against a possible neurotrophic role for GDNF is that mice homozygous null for GDNF and GFRα1 show no decrease in the number of SN dopamine neurons on the day of birth (Treanor et al. 1996; Pichel et al. 1996; Sanchez et al. 1996; Cacalano et al. 1998; Enomoto et al. 1998). However, these mice die shortly after birth because of developmental abnormalities of the kidney and enteric nervous system. Therefore, they die before much of the natural cell death event has occurred. Furthermore, none of these null mutations are temporally regulated, so compensatory changes may have taken place. These considerations offer ample grounds for not accepting the negative observations in the homozygous null mice as being definitive, insofar as a phenotype affecting the SN dopaminergic system is concerned.

To evaluate GDNF further as a possible neurotrophic factor for SN dopamine neurons, we assessed its ability to support their viability in a unique postnatal primary culture model (Rayport et al. 1992) established when these neurons would normally undergo their natural cell death event. We found that, among factors that had previously been reported to support mesencephalic dopamine neurons in embryonic culture, including brain-derived neurotrophic factor (BDNF), transforming growth factors (TGF) β1, 2, and 3, neurotrophin 3, b-fibroblast growth factor, TGFα, and epidermal growth factor, GDNF alone augmented survival and did so by suppressing apoptosis (Burke et al. 1998). To determine whether these observations could be confirmed in the in vivo context, we assessed the effect of GDNF injected into the striatum at PND2 on the level of natural cell death in SN dopamine neurons. We observed a 60% suppression of natural cell death by intrastriatal GDNF injection (Oo et al. 2003). To determine whether endogenous GDNF may play a role in regulating natural cell death, we injected GDNF-neutralizing antibodies into

the striatum. Two different neutralizing antibodies both induced natural cell death in dopamine neurons by 2–3 fold (Oo et al. 2003). We assessed the developmental dependence of these anti-GDNF antibodies to induce death and found that this effect was limited to the first postnatal week. Therefore, although our earlier lesion experiments had suggested that SN dopamine neurons were dependent on striatal target until PND14, i.e., throughout the first and second phases of natural cell death, dependence on GDNF was observed only through the first phase (Oo et al. 2003).

Thus, in its ability to regulate acutely the natural cell death event of SN dopamine neurons both in vitro and in vivo, GDNF fulfils many of the important criteria for a neurotrophic factor for these neurons. However, classic neurotrophic theory would also predict that a sustained increase in the supply of a limiting target-derived factor should augment the number of neurons that survive the natural cell death period. It is important to emphasize that an adequate test of this prediction requires a sustained increase in expression. Single intrastriatal injections of GDNF at PND2 have been shown not to have a lasting effect on the number of surviving dopamine neurons (Beck et al. 1996). However, single injections are unlikely to have a lasting effect, given that the natural cell death event takes place over a 2-week period. In order to achieve a sustained overexpression of GDNF in the target regions of the mesencephalic dopaminergic projections, we utilized a double transgenic approach as described by Mayford et al. (1996). We crossed mice transgenic for a CaMKII-tTA construct with mice transgenic for a BiTetO-LacZ-rGDNF construct. The double transgenic mice (CBLG-DT) demonstrated expression of LacZ specifically in the striatum (where it was most abundant), hippocampus and cortex, as previously described for CaMKII-tTA mice (Mayford et al. 1996; Yamamoto et al. 2000). It should be noted that selective overexpression of GDNF in these regions not only permits evaluation of GDNF specifically as a target-derived factor, but also avoids a detrimental effect of GDNF on SN dopamine neuron development when expressed within these neurons (Chun et al. 2002). The precise mechanism of this effect, which results in diminished numbers and size of dopamine neurons, is unknown, but it clearly necessitates a regionally specific overexpression of GDNF in target structures alone to avoid the deleterious effects of local nigral expression. The CBLG-DT mice overexpress GDNF in forebrain structures throughout the period of natural cell death (Kholodilov et al. 2004). Within the striatum, at the cellular level, β-galactosidase expression is strictly within medium striatal neurons, as it is for endogenous GDNF (Kholodilov et al. 2004).

Increased expression of GDNF within striatal medium-sized neurons throughout development leads to a 46% increase in the number of SN dopaminergic neurons surviving the first phase of natural cell death. This increase does not, however, persist into adulthood. Therefore, although striatal GDNF is both necessary and sufficient for the regulation of SN dopamine neuron survival during the first phase of natural cell death, it alone is not sufficient to lead to a lasting increase in their adult number. Some time between PND7 and adulthood, the number of these neurons must revert to their normal wildtype number. This does not occur as a "rebound" phenomenon, with an augmented level of natural cell death, during the second phase of death on PND14; we have indeed shown that, on the contrary, levels of apoptosis are reduced in the double transgenic mice on that day. Therefore, the time course and mechanism of "normalization" of the adult number of SN dopamine neurons in the CBLG-DT mice is unknown.

Just as there is no lasting increase in the adult number of SN dopamine neurons, there is no increase in nigral dopaminergic innervation of the striatum. We assessed morphologic features of TH-positive and dopamine transporter (DAT)-positive fibers, TH and vesicular monoamine transporter (VMAT2) protein expression, biochemical measures of dopamine and its metabolites, and physiologic measures of dopamine release and re-uptake and found no changes in the double transgenic mice.

The response of the ventral tegmental area (VTA) dopaminergic system to sustained overexpression of GDNF in targets was quite different from that of SN dopamine neurons. In the CBLG-DT mice, there was a 55% increase in the number of VTA dopamine neurons compared with wildtype controls in adult animals (Kholodilov et al. 2004). In addition, adult CBLG-DT mice demonstrated increased dopaminergic innervation of cortical regions, assessed by both TH and DAT-positive fiber staining. This morphologic phenotype was accompanied by a behavioral phenotype: CBLG-DT mice demonstrated an augmented motor activity response to amphetamine. Thus, there was a fundamental difference between the SN and VTA dopaminergic systems in their developmental response to GDNF expression in the target. We did not expect to observe this response in the VTA system in vivo, because in vitro, we had observed a greater ability of GDNF to suppress apoptosis in SN than VTA (Burke et al. 1998). The reason for this difference in effects of GDNF on VTA neurons between the in vitro and the in vivo contexts is unknown.

Thus, for the first phase of natural cell death in SN dopamine neurons, GDNF fulfils many of the criteria specified by classic neurotrophic theory. The ability of exogenous GDNF, when injected into the striatum, to reduce apoptosis and for overexpression to augment the surviving number of dopamine neurons clearly suggests that there is competition for GDNF during the first phase of cell death. The cellular basis for this competitive regulation strategy is unknown. Whether it is mediated at the synapse or by pre-synaptic neuronal activity is unknown. The way in which GDNF is released by postsynaptic neurons and what regulates its release are also unknown.

The question arises regarding what factors, after the first phase of natural cell death, are important regulators of SN dopamine neuron survival. In the absence of an increase in such factors, increased target GDNF alone is unable to change the number of surviving dopamine neurons. One

obvious possibility is that the abundance of the receptor, GFRα1, may become limiting. Whereas such regulation may occur at the level of the SN, autonomous to dopamine neurons, there is an alternate possibility that it may occur in a non-cell autonomous fashion at the level of striatal target. The possibility that GFRα1 may act in trans to influence, in a non-cell autonomous fashion, incoming projection systems that express the signaling kinase Ret has been proposed by Yu et al. (1998), as they have observed a discrepancy in some developing regions between GFRα1 and Ret mRNA expression. They have noted that often regions that express abundant GFRα1 mRNA, but little Ret, are the targets of systems abundant in Ret expression (Yu et al. 1998). In support of the concept that GFRα1 may act in a non-cell autonomous fashion to regulate neural development, Paratcha et al. (2001) have demonstrated that GFRα1 can be released by neurons and modulate neurite outgrowth, guidance, and neuron survival. This group of investigators has also shown that, in the presence of saturating concentrations of GDNF, immobilized exogenous GFRα1 acting in trans can influence the strength and direction of neurite outgrowth (Ledda et al. 2002). We have found that GFRα1 is expressed not only in SNpc, as previously shown, but also in striatal medium-sized neurons (J. Cho et al., submitted). In the striatum, it is maximally expressed between PND10 and PND14. In this location, it theoretically could act in trans to regulate the development of the nigro-striatal projection and the viability of SN dopamine neurons. This will be an important future area of investigation.

Other neurotrophic factors affecting the development of SN dopamine neurons

Among the other members of the GDNF family of ligands, neurturin has been considered as a possible neurotrophic factor for dopamine neurons. Neurturin was originally cloned by virtue of its ability to support the survival of sympathetic neurons in culture (Kotzbauer et al. 1996). It has been shown in many studies both to protect and to restore SN dopamine neurons in vitro and in vivo (Akerud et al. 1999; Horger et al. 1998; Oiwa et al. 2002). Its highest levels of mRNA expression in the striatum are at PND15 (Akerud et al. 1999), suggesting that perhaps it plays a role during the second phase of natural cell death. We have found, however, that patterns of neurturin mRNA expression are not highly suggestive of a role as a target-derived factor for SN dopamine neurons. Neurturin mRNA is much more abundant in SN than it is in striatum (Cho et al. 2004), unlike GDNF. One may consider therefore that, rather than serving as a target-derived factor, neurturin may serve in a local nigral autocrine or paracrine role. However, no developmental regulation of neurturin expression occurs within the SN (Cho et al. 2004). In addition, although the neurturin receptor, GFRα2, is highly expressed in the SN and developmentally regulated (Cho et al. 2004), it does not appear to co-localize with SN dopamine neurons (Horger et al. 1998). Thus, the precise physiologic role of neurturin, if any, in regulating the normal development of dopamine neurons remains to be defined.

Although many neurotrophic factors have been reported to have effects on the development of SN dopamine neurons in embryonic primary culture, for the purposes of this review, we will only consider factors that have been reported to have effects on development in vivo.

BDNF, first purified in 1982 (Barde et al. 1982) and cloned in 1989 (Leibrock et al. 1989), was the first purified neurotrophic factor identified as supporting the survival of mesencephalic dopamine neurons in the absence of glial cells (Hyman et al. 1991). Supranigral infusion of BDNF was subsequently shown to reverse behavioral and biochemical deficits induced by intrastriatal infusion of 6OHDA (Altar et al. 1994). However, BDNF did not protect mesencephalic dopamine neurons from axotomy (Knusel et al. 1992; Lapchak et al. 1993). In relation to the development of SN dopamine neurons, BDNF is unlikely to serve as a striatal target-derived factor. Although the BDNF receptor, TrkB, is expressed within SNpc neurons (Lindsay 1993), BDNF mRNA is not detectable in either the newborn or adult striatum (Maisonpierre et al. 1990). We have shown that intrastriatal injection of BDNF has no effect on levels of natural cell death among dopamine neurons of the SN (Oo et al. 2003). Although BDNF is unlikely to serve as a target-derived factor for SN dopamine neurons, it is possible that it regulates their development via an anterograde influence. It is well established that anterograde and retrograde influences can regulate the magnitude of natural cell death (Linden 1994). There is growing evidence that BDNF may be transported anterogradely within the central nervous system (Altar and DiStefano 1998). Mice expressing a transgene in which BDNF expression is driven by the dopamine β-hydroxylase (DBH) promoter have over a 50% increase in their adult number of SN dopamine neurons (Alonso-Vanegas et al. 1999). The control of BDNF expression by the DBH promoter would result in the selective overexpression of BDNF by noradrenergic neurons. These investigators postulate that an adrenergic afferent projection to the SN conveys an increased local release of BDNF in these animals; this supresses natural cell death and results in an increased number of neurons surviving into adulthood (Alonso-Vanegas et al. 1999). This interesting hypothesis will need to be explored by direct assessment of levels of apoptotic death among dopamine neurons in these animals.

TGFα mRNA is highly expressed in the striatum (Lazar and Blum 1992; Wilcox and Derynck 1988) and reaches its maximal level of expression on PND1 (Lazar and Blum 1992), and so it has been evaluated for a possible role as a target-derived neurotrophic factor for SN dopamine neurons. Alexi and Hefti (1993) have demonstrated that it is capable of supporting the differentiation and survival of embryonic mesencephalic dopamine neurons. We have not found, however, that it is capable of suppressing

apoptosis in SN dopamine neurons in postnatal primary culture (Burke et al. 1998). Nevertheless, TGFα remains of interest in relation to the development of SN dopamine neurons, because TGFα homozygous null mice have only about 50% the number of these neurons as wildtype controls (Blum 1998). This difference is attributable neither to diminished phenotype expression, because it has also been observed for counts of Nissl-stained profiles, nor to an accentuation of natural cell death, because the difference is present at PND1. The latter observation would suggest that TGFα influences the prenatal ontogeny of dopamine neurons, during either their proliferation or successful migration.

Do adult dopamine neurons of the SN depend on striatal target-derived trophic support for viability?

One hypothesis for the death of SN dopamine neurons in Parkinson's disease and related disorders is that the disease process begins not primarily in the cell bodies of these neurons, but instead either in postsynaptic striatal neurons or in the axon terminals of dopamine neurons, such that there is a failure of target-derived retrograde support, leading ultimately to the death of dopamine neurons as a secondary event. Although we have shown that such a scenario can indeed occur in the rodent during development, the question arises as to whether such an event might also occur in the adult brain. There is little evidence that this occurs; on the contrary, much evidence suggests it does not. We have shown that a postnatal striatal axon-sparing lesion will induce death of SN dopamine neurons through PND14, but thereafter it does not (Kelly and Burke 1996). In adult rats, an extensive striatal axon-sparing lesion results in transneuronal degeneration of neurons within the SN pars reticulata, but not of the SNpc (Stefanis and Burke 1996; Krammer 1980; Pasinetti et al. 1991; Saji and Reis 1987). Björklund and coinvestigators have shown that such a lesion leads to the atrophy, but not loss, of SN dopamine neurons (Lundberg et al. 1994). In a similar vein, we have established that, whereas intrastriatal injection of anti-GDNF neutralizing antibodies induces death in SN dopamine neurons during the first postnatal week, thereafter it does not; there is no evidence of a persistent dependence on striatal GDNF in more mature animals (Oo et al. 2003). Therefore, we propose that the death of SN dopamine neurons in Parkinson's disease is not attributable to the failure of target support. Nevertheless, whatever the proximate causes of neuron death in the disease, there is growing evidence that the molecular pathways of cell death initiated by these causes are closely related to those mediating programmed cell death in the developmental setting. Furthermore, the cellular events that will be required successfully to replace SN dopamine neurons and accurately guide them to their targets, will presumably recapitulate normal target-regulated developmental events. Therefore, it will be important to understand these processes in order to develop both neuroprotective and symptomatic treatments for Parkinson's disease.

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